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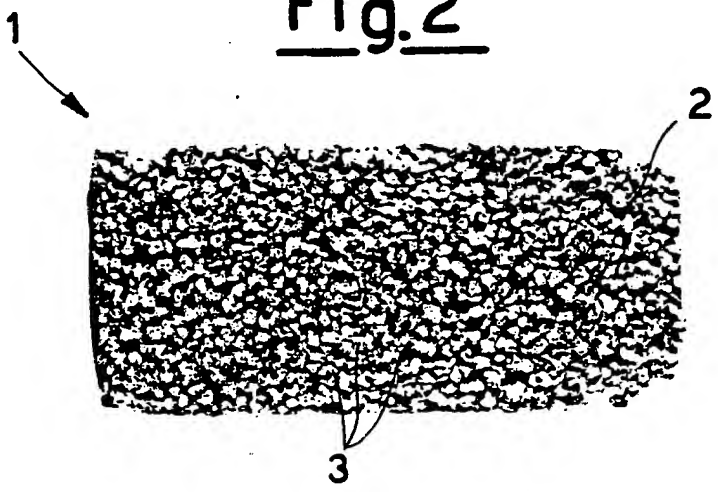
(54) Surgical aid endowed with osteotropic activity.

(57) The surgical aid endowed with osteotropic activity comprises a base and a suspended substance constituting the active element disperded in the base. The active element is constituted by granular, ceramic hydroxyapatite, and the base is gelatine of pharmacologic grade in a pure state, to which glycerol is possibly added. The aid is used in anhydrous phase, and as thin sheets and filaments, the flexibility of which increases with increasing percentage of glycerol in the base.

Fig.2



EP 0 329 239 A2

SURGICAL AID ENDOWED WITH OSTEOTROPIC ACTIVITY

The present invention is concerned with a surgical aid performing an osteotropic action.

During the operations of orthopedic surgery, odontiatric surgery, and in parodontium pathology, the need usually occurs for:

- filling hollows,
- removing defects and differences in level of bony planes,
- reconstructing portions of bony tissue, by operating in such a way as to favour a rapid restoration of the morphology of the bony segment the tissue of which suffered lesions or underwent modifications.

In order to meet the above needs, the use of various types of hydroxy-apatite in presently known.

From an operative viewpoint, the granules of hydroxy-apatite are manually deposited on the region to be treated, so that they may behave as an osteotropic element. Therefore, after healing, the hydroxy-apatite granules are incorporated in an at all compatile way inside the bony tissue formed.

However, the operation of deposition of the granules of hydroxy-apatite calls for a great skill by the surgeon.

Such a great skill is essential for the surgeon, in order that he can correctly meter the necessary amount of hydroxy-apatite, and to house it in the nearby of the lesion in such a way that, after the wound being sutured, the granules of said hydroxy-apatite get not dispersed during the surgical healing process.

If hydroxy-apatite is not correctly metered, but, for example, a lower than optimum amount thereof is metered, after the healing a satisfactory clinic result is not obtained. If, for example, hydroxy-apatite is metered in a larger amount than as necessary, the healing process ends anyway with a poor clinic result; moreover, the probability is higher, that the granules get dispersed in correspondence of the bony lesion, therefore moreover causing a waste of a particularly expensive material.

Said considerable skill is furthermore required from the surgeon, in order that he may correctly insert the granules of hydroxy-apatite inside hollows which are difficult to reach, extemporarily creating, by resorting to makeshift means, small instruments or guides, which are capable of fulfilling the surgical requirements.

Particularly difficult situations have to be frequently confronted with in case of odontiatric operations, during which the bony tissue has to be integrated in particularly narrow areas.

The purpose of the present invention is of providing a surgical aid performing an osteotropic action, which is capable of obviating the above-said drawbacks.

Such purposes are achieved by a surgical aid endowed with an osteotropic activity, characterized in that it comprises a base and a suspended substance, which constitutes the active element, with said base being constituted by gelatine of pharmacologic grade, in a pure state, and the suspended matter being constituted by ceramic hydroxy-apatite dispersed in the base.

The advantages attained by means of the present invention essentially consist in that:

- it can be easily used, in that the metering of the granules of hydroxy-apatite is not required;
- it makes it possible the only necessary amount to be used, with the operation costs being reduced;
- it can be easily and stably applied (in that it has a firm structure, not a simply granular structure), without resorting to special equipment pieces being necessary;
- it is easily anchored in the area in which it is necessary, with no risks that it may get dispersed or displaced during the clinic healing process;
- it is ductile and malleable, with the possibility of use of the product being hence increased.

The invention is illustrated for merely exemplifying, and non-limitative, purposes, in the figures of the hereto attached drawing tables.

Figure 1 shows a surgical aid according to the present invention, in a form which makes it possible it to be immediately used, in that said surgical aid is given the shape of a laminar structure, with a high density of granules of active material;

Figure 2 shows a surgical aid according to the present invention, also in a form which makes it possible it to be immediately used, in that said surgical aid is given the shape of a laminar structure with a low density of granules of active material.

Referring to the above cited figures, the surgical aid according to the present invention, generally indicated by the reference numeral 1, comprises a base and a suspended substance, which constitutes the active element. The base 2 is constituted by gelatine of pharmacologic grade, in a pure state (hereinunder simply referred to as the "gel"), and the suspended matter is constituted by granules 3 of ceramic hydroxy-

apatite dispersed throughout the base by kneading.

In order to give the product a certain plasticity, glycerol is added.

The optimum chemical composition of the surgical aid in hydrate phase is indicatively as follows:

- hydroxy-apatite	61.35%
- gel	13.56%
- glycerol	12.65%
- water	12.44%

The so-obtained product is dried by evaporation under vacuum, with it being heated to a temperature not higher than 40 ° C, in order to prevent the gel from undergoing any modifications.

The end product obtained after water evaporation, and therefore of the anhydrous phase, is as follows:

- hydroxy-apatite	70.00%
- gel	15.05%
- glycerol	14.04%
- water	0.91%

The so-obtained product, which is malleable and ductile, is mechanically shaped so as to give it the shape of very thin sheets, small rods, filaments, or any other shapes which make it possible it to be easily and promptly used after being sterilized by means of gamma rays.

The hydroxy-apatite used in order to form the surgical aid is in the form of granules having a diameter indicatively comprised within the range of from 0.5 to 1.8 mm.

The characteristics of elasticity and flexibility are mainly influenced by the presence of glycerol; more precisely, with increasing glycerol percentage, the characteristics of flexibility and elasticity increase; with the percentages of glycerol gradually decreasing, a substantially more and more rigid product is obtained.

Therefore, a change in glycerol percentage relatively to the gel percentage is provided, which is comprised within the range of about ±4%.

The osteotropic aid shown in Figure 1 is of the laminar type, with a high granular density, whilst the aid shown in Figure 2 is of the type with a low granular density.

The application of the aid shown in Figure 1 is particularly indicated for the reconstruction of bone defects in some parodontium illnesses and the like; whilst the aid shown in Figure 2 is used after various resections of benign tumors, after the removal of bony tissue owing to traumatic causes, in the substitution of special endoprostheses, and the like. The granules 3 of hydroxy-apatite retained by the base 2 of interstitial gel are visible.

For the application, the surgical aid in anhydrous phase is used cut into pieces of the required size by means of a common pair of scissors, or suitably dimensioned pre-formed pieces are used.

The gel prevents the granules of hydroxy-apatite 3 from getting dispersed both during the operation, and during the clinic healing process.

Therefore, the granules of hydroxy-apatite are progressively encapsulated inside the bony tissue during the step of cicatricial rebuilding.

The hydrate gel is progressively dissolved and metabolized.

Claims

1. Surgical aid endowed with an osteotropic activity, characterized in that it comprises a base and a suspended substance, which constitutes the active element, with said base being constituted by gelatine of pharmacologic grade, in a pure state, and the suspended matter being constituted by ceramic hydroxy-apatite dispersed in the base.

2. Aid according to claim 1, characterized in that said aid contains glycerol.

3. Aid according to claim 2, characterized in that two phases thereof are provided: an anhydrous phase and a hydrate phase, with said anhydrous phase substantially comprising:

- hydroxy-apatite	70.00%
- gel	15.05%
- glycerol	14.04%
- water	0.91%

and said hydrate phase substantially comprising:

- hydroxy-apatite	61.35%
- gel	13.56%
- glycerol	12.65%
- water	12.44%

4. Aid according to claim 3, characterized in that the percentage of glycerol is variable relatively to the gel percentage, by a value of ±4%.

5. Aid according to claim 3, characterized in that hydroxy-apatite is as granules, the diameter of which is comprised within the range of from 0.5 to 1.8 mm.

6. Aid according to claim 3, characterized in that in the anhydrous state it is both elastic and flexible.

7. Process for the production of a surgical aid, characterized in that it comprises

- a first step during which the ingredients are mixed, with a hydrate phase being obtained eventually, the percentages of which are identical to those as stated in claim 3,
- a second step during which a drying is carried out, in order to obtain an anhydrous phase,
- a third sted during which the product is given the shape of thin sheets and/or filaments,
- a fourth step of sterilization and packaging.

8. Process according to claim 6, characterized in that the drying step is carried out at a temperature lower than 40° C, and under vacuum conditions.

9. Process according to claim 6, characterized in that the sterilization is carried out by irradiation with gamma rays.

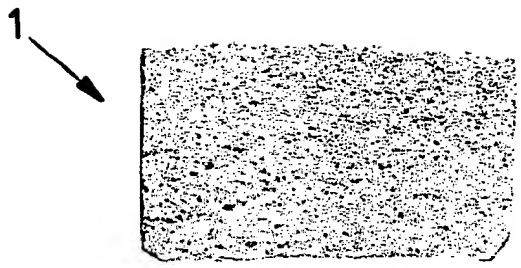


Fig.1

Fig.2

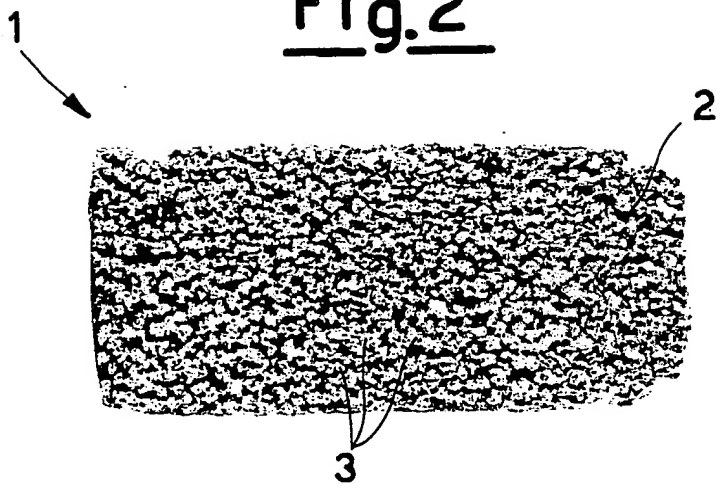


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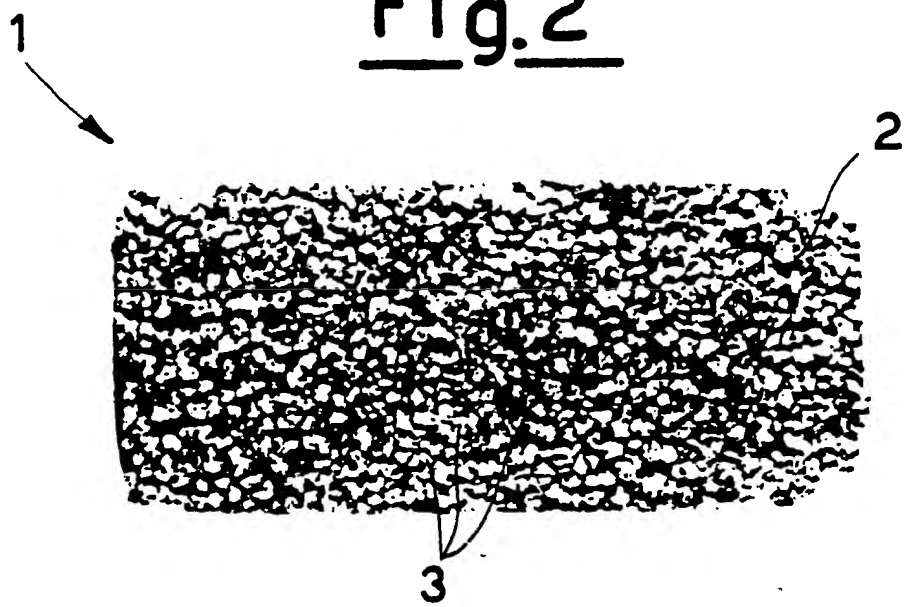
Fig.2



EP 0 329 239 A3

European Patent Office

EUROPEAN SEARCH REPORT

Application Number

EP 89 20 0330

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.4)
X	WO-A-8 601 113 (G. BRINKS) * Claims 1-2; page 2, line 26 - page 3, line 7; page 3, lines 21-35; pae 4, lines 19-22 *	1,3,5	A 61 L 27/00 A 61 L 25/00 A 61 K 6/00
X	US-A-4 349 470 (O. BATTISTA) * Example 5 *	1,5	
Y	US-A-4 357 935 (W. FRANTZICH) * Column 1, lines 29-36 *	9	
Y,P	EP-A-0 270 254 (COLLAGEN CORP.) * Claims *	9	
A	EP-A-0 147 021 (ED. GEISTLICH SOEHNE)		

TECHNICAL FIELDS SEARCHED (Int. Cl.4)

A 61 L
A 61 K

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	17-05-1990	COUSINS-VAN STEEN G.I.L.

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

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The microorganism(s) has (have) been deposited with ATCC-HB under numbers 10099 and 10098.

(54) **Bone-inducing protein.**

(57) An osteogenically active protein, having the sequence (H_2N)-Ala-Lys-Tyr-Asn-Lys-Ile-Lys-Ser-Arg-Gly-Ile-Lys-Ala-Asn-Thr-Phe-Lys-Lys-Leu-His-Asn-Leu-Ser-Phe-Leu-Tyr-Leu-Asp-His-Asn-Ala-Leu-Glu-Ser-Val-Pro-Leu-Asn-Leu-Pro-Glu-Ser-Leu-Arg-Val-Ile-His-Leu-Gln-Phe-Asn-Asn-Ile-Thr-Ser-Ile-Thr-As p-Asp-Thr-Phe-Cys-Lys-Ala-Asn-Asp-Thr-Ser-Tyr-Ile-Arg-Asp-Arg-Ile-Glu-Glu-Ile-Arg-Leu-Glu-Gly-Asn-Pro-Val-Ile-Leu-Gly-Lys-His-Pro-Asn-Ser-Phe-Ile-Cys-Leu-Lys-Arg-Leu-Pro-Ile-Gly-Ser-Tyr-Ile-Asp-(COOH),
and substantially pure polypeptides that are substantially equivalent and substantially homologous thereto is disclosed. Pharmaceutical compositions containing these polypeptides and methods to use them are also disclosed.

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Description

BONE-INDUCING PROTEIN

Technical Field

The present invention relates to protein chemistry and osteoplasty. More particularly, it relates to a protein that induces bone growth, implant and pharmaceutical compositions containing that protein, methods for promoting bone growth using such compositions, methods for stimulating bone marrow progenitor cells to divide and differentiate into bone marrow cells, and methods for treating diseases associated with dysfunction/malfunction of bone generation and/or bone resorption, such as osteoporosis.

Background Art

It has been established that bone contains materials which can stimulate the formation of new bone when placed in contact with living systems. (Urist, M. R., Clin Orthop (1968) 56:37; Science (1965) 150:893; Reddi, A. H., et al., Proc Natl Acad Sci (USA) (1972) 69:1601.) Attempts have been made to purify whatever factors are responsible for this activity. A "bone morphogenic protein" (BMP) was extracted from demineralized bone using urea or guanidine hydrochloride and reprecipitated according to the disclosures in U.S. Patents Nos. 4,294,753 and 4,455,256 to Urist. Urist subsequently reported (Urist, M. R., Clin Orthop Rel Res (1982) 162:219) that ion exchange purification of this crude protein mixture yielded an activity which was unadsorbed to carboxymethyl cellulose resin (CMC) at pH 4.8. Urist's reports in Science (1983) 220:680-685 and Proc Natl Acad Science (USA) (1984) 81:371-375 describe BMPs having molecular weights of 17,500 and 18,500 daltons. Urist's patent publication, EPA Publication No. 0212474, describes BMP fragments of 4,000 to 7,000 daltons obtained by limited proteolysis of BMP.

U.S. Patent No. 4,608,199 describes a bone-derived protein of 30,000-32,000 daltons. The protein is described as being water soluble and having no affinity for concanavalin A (ConA).

WO 88/00205 reports four proteins, designated BMP-1, BMP-2 Class I, BMP-2 Class II and BMP-3, that are alleged to have osteogenic activity by themselves or in combination with other factors. Sequences are provided for each of these proteins which show no homology to the sequence (see below) of the osteogenic protein of the present invention.

U.S. 4,434,094 to Seyedin and Thomas reported the partial purification of a bone generation-stimulating, bone-derived protein by extraction with chaotropic agents, fractionation on anion and cation exchange columns, and recovery of the activity from a fraction adsorbed to CMC at pH 4.8. This new protein fraction was termed "osteogenic factor" (OF) and was characterized as having a molecular weight below about 30,000 daltons.

Commonly owned U.S. Patent No. 4,774,332 describes two proteins that were purified to homogeneity using a purification procedure that is similar in part to that disclosed in U.S. 4,434,094. Those two proteins eluted from CMC at about a 150-200 mM NaCl gradient. These two proteins were originally called cartilage-inducing factor (CIF) A and CIF B. CIF A was subsequently found to be identical to a previously identified protein called transforming growth factor beta (TGF-beta). CIF B has been found to be a novel form of TGF-beta and is now known as TGF-beta2.

Commonly owned U.S. Patent No. 4,627,982 concerns a partially purified bone-inducing factor present in the CMC-bound fraction of U.S. 4,434,094 that elutes in the portion of the NaCl gradient below that in which the major portions of CIF A and CIF B elute (i.e., below about 150 mM NaCl). The present invention relates to the identification of the active ingredient of that fraction. In this regard, at the time that patent was filed it was not known whether the bone-inducing activity was attributable to a single protein or a plurality of proteins acting in concert. Identification of the protein(s) responsible for bone-inducing activity was complicated by the large number of proteins in the fraction (estimated to be several hundred), the lack of a conclusive in vitro assay for bone-inducing activity, and extensive difficulty in isolating the active protein from other proteins in the fraction. Indeed, it has taken applicants approximately three years of effort--in which a variety of protein fractionation procedures were attempted--to obtain the bone-inducing protein from the CMC-bound fraction at a level of purity at which it could be sequenced and identified as being responsible for the activity observed in the crude fraction.

As discussed in detail below, the bone-inducing activity of the fraction has been found to be attributable to a glycoprotein component having a variable (apparently due to variation in glycosylation) molecular weight (in the glycosylated state) of approximately 20,000-28,000 daltons as determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis. Amino acid sequencing of the active component indicates it is composed of a protein that has a sequence different from any previously reported sequence.

Disclosure of the Invention

The invention relates to a substantially pure polypeptide that induces bone formation in vivo in mammals.

Accordingly, one aspect of the invention is a substantially pure osteogenically active polypeptide haying an internal sequence selected from the group consisting of

a) -Lys-Tyr-Asn-Lys-Ile-Lys-Ser-Arg-Glu-Ile-Lys-Ala-Asn-Thr-Phe-Lys-Lys-Leu-His-Asn-Leu-Ser-Phe-Leu-Tyr-Leu-Asp-His-Asn-Ala-Leu-Glu-;

b) -Leu-His-Asn-Leu-Ser-Phe-Leu-Tyr-Leu-Asp-His-Asn-Ala-Leu-Glu-Ser-Val-Pro-Leu-

Asn-Leu-Pro-Glu-;

c) -Ser-Leu-Arg-Val-Ile-His-Leu-Gln-Phe-Asn-Asn-Ile-Thr-Ser-Ile-Thr-Asp-Asp-Thr-Phe-Cys-Lys-Ala-;

d) -Ala-Asn-Asp-Thr-Ser-Tyr-Ile-Arg-Asp-Arg-Ile-Glu-Glu-Ile-Arg-Leu-Glu-Gly-Asn-Pro-Val-Ile-;

e) -Gly-Asn-Pro-Val-Ile-Leu-Gly-Lys-His-Pro-Asn-Ser-Phe-Ile-Cys-Leu-Lys-Arg-Leu-Pro-Ile-Gly-Ser-Tyr-; and/or a carboxy terminal sequence as follows

-Arg-Leu-Pro-Ile-Gly-Ser-Tyr-Ile-Asp-(COOH),

and substantially pure polypeptides that are substantially equivalent and substantially homologous thereto.

Another aspect of the invention is a substantially pure osteogenically active polypeptide having the following amino acid sequence:

(H_2N)-Ala-Lys-Tyr-Asn-Lys-Ile-Lys-Ser-Arg-Gly-Ile-Lys-Ala-Asn-Thr-Phe-Lys-Lys-Leu-His-Asn-Leu-Ser-Phe-Leu-Tyr-Leu-Asp-His-Asn-Ala-Leu-Glu-Ser-Val-Pro-Leu-Asn-Leu-Pro-Glu-Ser-Leu-Arg-Val-Ile-His-Leu-Gln-Phe-Asn-Asn-Ile-Thr-Ser-Ile-Thr-Asp-Asp-Thr-Phe-Cys-Lys-Ala-Asn-Asp-Thr-Ser-Tyr-Ile-Arg-Asp-Arg-Ile-Glu-Glu-Ile-Arg-Leu-Glu-Gly-Asn-Pro-Val-Ile-Leu-Gly-Lys-His-Pro-Asn-Ser-Phe-Ile-Cys-Leu-Lys-Arg-Leu-Pro-Ile-Gly-Ser-Tyr-Ile-Asp-(COOH),

and substantially pure polypeptides that are substantially equivalent and substantially homologous thereto.

Another aspect of the invention is a composition for inducing bone formation or bone marrow cell production in vivo comprising (a) an effective amount of one or more of the above-described osteogenically active polypeptides and (b) an effective amount of TGF-beta combined with (c) a pharmaceutically acceptable carrier.

Another aspect of the invention is a method of inducing bone formation in vivo at a desired site comprising implanting the above described composition in a mammal at said site.

Yet another aspect of the invention is a method of inducing bone marrow cell production in a living mammal comprising administering systemically to the mammal an effective amount of one or more of the above described polypeptides.

Another aspect of the invention is a method of treating an individual for a condition characterized by insufficient bone formation and/or undesired bone resorption comprising administering systemically to the individual an effective amount of one or more of the above-described polypeptides.

Still another aspect of the invention is an improvement in a method for treating a living mammal for cancer of the hematopoietic system wherein the mammal is subjected to irradiation to kill neoplastic hematopoietic cells the improvement comprising administering systemically to the mammal prior to irradiation a sufficient amount of TGF-beta to suppress hematopoietic stem cell division and administering systemically to the mammal after irradiation a sufficient amount of one or more of the above described polypeptides to stimulate hematopoietic stem cell division.

Another aspect is a method for isolating a substantially pure osteogenically active protein composition from demineralized bone comprising:

(a) extracting demineralized bone with a chaotropic (dissociative) extractant that solubilizes nonfibrous proteins;

(b) subjecting the extract from step (a) to gel filtration to recover a fraction containing proteins of molecular weight of approximately 20,000-36,000 daltons;

(c) adsorbing the fraction from step (b) onto a carboxymethyl cellulose cation exchanger at approximately pH 4.5-5.5 under denaturing conditions;

(d) eluting a fraction from the cation exchanger with a sodium chloride gradient of about 10 mM to about 150 mM;

(e) adsorbing the eluate of step (d) onto a cross-linked ConA column;

(f) eluting bound protein from the column of step (e);

(g) adsorbing the eluate of step (f) onto a heparin-sepharose column;

(h) eluting bound protein from the column of step (g); and

(i) chromatographing the eluate of step (h) on an RP-HPLC column using a trifluoroacetic acid- acetonitrile system and recovering the substantially pure osteogenically active protein composition as the fraction eluting from the column at approximately 42-45% acetonitrile.

Further aspects of the invention are recombinant materials (i.e., recombinant DNA, recombinant vectors, and recombinant cells or microorganisms) and processes for producing the osteogenic proteins of the invention.

Brief Description of the Drawings

In the drawings:

Figure 1 is a flow chart of the process that was used to isolate osteogenic protein from demineralized bovine bone.

Figure 2 is a graph of the optical densities (absorbances at 280 nm) of the gel filtration fractions of the gel filtration fractions of the example (©C).

Figure 3 is a graph of the optical densities (absorbances at 280 nm) of eluate fractions from the preparative ion exchange chromatography of the example (©D).

Figure 4 is a graph of the optical densities (absorbances at 280 nm) of eluate fractions from the cross-linked ConA chromatography step of the example (©E);

Figure 5 is a graph of the optical densities (absorbances at 280 nm) of eluate fractions from the heparin-sepharose chromatography step of the example (©F); (absorbances at 230 nm) of the gradient fractions from the

Figure 6 is a graph of the optical densities (absorbances at 230 nm) of the gradient fractions from the C18-RP-HPLC chromatography step of the example (©G);

Figure 7 is a table showing results of amino acid sequencing of the osteogenically active

isolate of the invention and locations of the sequenced fragments in the overall sequence.

Figure 8 is a photograph of an autoradiograph of SDS-PAGE analyses of the purified osteogenic protein that are described in the example (©H) (lanes A and C show glycosylated protein; lanes B and D show enzymatically deglycosylated protein).

Figure 9 is a bar graph showing the results of the assays described in the example (©I.3 and I.4).

Figure 10 is a bar graph of the results of the assays described in ©J.1. of the examples.

Figure 11 is a graph of the results of the tests described in ©J.3. of the examples.

Modes of Carrying Out the Invention

Isolation of Bone-Inducing Protein from Bone

In view of the showing that bone inductive proteins from human, monkey, bovine and rat are nonspecies specific in their ability to produce endochondral bone in xenogeneic implants (Sampath, T.K., et al, Proc Natl Acad Sci (USA) (1983) 80:6591) it is believed that the bovine protein described herein has been highly conserved among mammalian species--i.e., corresponding bone-inducing proteins from different mammalian species (herein called "species analogs") will have substantially homologous amino acid sequences that vary from the bovine protein, if at all, in one or more amino acid residue additions, deletions or substitutions and/or substantially similar glycosylation patterns that do not affect the nonspecies-specific ability of the molecule to induce bone formation. In this regard, the terms "substantially equivalent" and "substantially homologous" are intended to mean proteins, regardless of species or method of preparation, that have the same amino acid sequence as the bovine osteogenic protein described in the examples and proteins of similar but different amino acid sequence, which difference(s) does not affect nonspecies-specific endochondral bone- inducing activity adversely. The amino acid sequences of such "substantially homologous" proteins will usually be at least 50% homologous, more usually at least 80% homologous, and preferably at least 90% homologous to the bovine sequence described herein. Accordingly, such proteins may be derived from bone of diverse mammalian origin or synthesized using recombinant DNA procedures. The term is intended to include muteins or analogs of the native protein that are altered in manners known in the art, such as by substitution of cysteines that are not essential for activity with neutral (uncharged) amino acids to avoid improper disulfide bonding, by substitution or elimination of residues in the asparagine-linked glycosylation sites of the proteins to alter glycosylation patterns, by substitution of methionines that are not necessary for activity to make the molecules less susceptible to oxidation, by chemical modification of one or more residues, or by elimination or alteration of side-chain sugars. The source of protein prepared by purification from native sources is advantageously porcine or bovine long bone because of its ready availability.

The process for isolating the osteogenic protein from bone is as follows. The bone is first cleaned using mechanical or abrasive techniques, fragmented, and further washed with, for example, dilute aqueous acid preferably at low temperature. The bone is then demineralized by removal of the calcium phosphates in their various forms, usually by extraction with stronger acid. These techniques are understood in the art, and are disclosed, for example, in U.S. 4,434,094. The resulting preparation, a demineralized bone, is the starting material for the preparation of the claimed osteogenic protein from native sources.

The initial extraction is designed to remove the nonfibrous (e.g., noncollagenous) proteins from the demineralized bone. This can be done with the use of chaotropic agents such as guanidine hydrochloride (at least about 4 molar), urea (8 molar) plus salt, or sodium dodecylsulfate (at least about 1% by volume) or such other chaotropic agents as are known in the art (Termine et al., J Biol Chem (1980) 255:9760-0772; and Sajera and Hascall, J Biol Chem (1969) 244:77-87 and 2384-2396). The extraction is preferably carried out at reduced temperatures to reduce the likelihood of digestion or denaturation of the extracted protein. A protease inhibitor may be added to the extractant, if desired. The pH of the medium depends upon the extractant selected. The process of extraction generally takes on the order of about 4 hr to 1 day.

After extraction, the extractant may be removed by suitable means such as dialysis against water, preceded by concentration by ultrafiltration if desired. Salts can also be removed by controlled electrophoresis, or by molecular sieving, or by any other means known in the art. It is also preferred to maintain a low temperature during this process so as to minimize denaturation of the proteins. Alternatively, the extractant chaotropic agent need not be removed, but rather the solution need only be concentrated, for example, by ultrafiltration.

The extract, dissolved or redissolved in chaotropic agent, is subjected to gel filtration to obtain fractions of molecular weight in the range of about 20,000 to 36,000 daltons. Gel sizing is done using standard techniques, preferably on a Sephacryl S-200 column at room (10°C-25°C) temperature.

The sized fraction is then subjected to ion exchange chromatography using CMC at approximately pH 4.5-5.2 preferably about 4.8, in the presence of a nonionic chaotropic agent such as 6 M urea. Other cation exchangers may be used, including those derived from polyacrylamide and cross-linked dextran; however cellulosic cation exchangers are preferred. Of course, as in any ion exchange procedure, the solution must be freed of competing ions before application to the column. The factor is adsorbed on the column and is eluted in an increasing salt concentration gradient in the range of about 10 mM to about 150 mM. This fraction is designated "CMB-1" for convenience.

CMB-1 is lyophilized and the dry CMB-1 is dissolved in aqueous sodium deoxycholate (DOC), pH 8.0. This solution is affinity chromatographed on

an equilibrated column of ConA cross-linked to resin. The ConA-bound material is eluted from the resin with aqueous DOC containing a displacement carbohydrate. This fraction is designated "CAB-1" for convenience.

CAB-1 is reequilibrated for heparin-sepharose chromatography by desalting on a GH-25 column equilibrated on heparin-sepharose buffer, 6 M urea, 0.1 M NaCl, 50 mM Tris-HCl pH 7.2. The desalted fraction is loaded into a heparin-sepharose column. After washing, bound material is eluted from the column using the same buffer at a 0.5 M NaCl salt concentration. The resulting eluate is designated "HSB-1" for convenience.

HSB-1 is diluted and adjusted to pH 2 and loaded onto a C18-RP-HPLC column. Bound proteins were gradient eluted from the column using a solvent consisting of 90% acetonitrile in 0.1% aqueous TFA (Solvent B). The osteogenic protein of the invention elutes at approximately 47-50% of solvent B (42-45% acetonitrile) by volume.

Proteins eluted by the C18 chromatography were iodinated by the chloramine-T method. Analysis of the fraction by SDS-PAGE and autoradiography shows a major broad band at 20,000 to 28,000 daltons comprising the osteogenic protein. The "smearing" of the protein is believed to mainly be the result of heterogeneity in the glycosylation of the molecule or perhaps variable post-translational modification or proteolytic degradation. After enzymatic or chemical deglycosylation, SDS-PAGE analysis of the protein gives a single band of approximately 9600 daltons. Reduction of the deglycosylated protein with dithiothreitol does not affect its migration.

Initial amino acid sequence analysis of the glycosylated protein yielded the following internal sequence in the N-terminal portion of the protein:
-Lys-Tyr-Asn-Lys-Ile-Lys-Ser-Arg-Gly-Ile-Lys-Ala-
Asn-Thr-Phe-Lys-Lys-Leu-His-Asn-Leu-Ser-Phe-
X-Tyr-Thr-Asp-His-Asn-Ala-Leu-Glu-

The initial amino acid (Lys) in the above sequence is nearest to the N-terminal. Initially, the nature of the signal obtained for the residue designated X did not permit this residue to be identified. Repeated sequencing and sequencing of endoproteinase Lys-C (an enzyme that cleaves proteins at Lys residues) and endoproteinase Glu-C (an enzyme that cleaves proteins at Glu residues) digests have revealed that the above sequence is preceded by an Ala residue which is the N-terminus, that the residue designated X is Leu, that the second Thr residue (the 26th residue in the above sequence) was incorrect and that this residue is actually a Leu residue, and that the isolate consists of a protein of approximately 106 amino acids. Figure 7 provides a summary of these sequence analyses. The symbol "CHO" designates a carbohydrate substituent. The symbol "COOH" represents a carboxyl group and designates the carboxy terminus. The first column (on the left) provides the sequence analysis of the N-terminal fragment described above. The second, fourth, and sixth columns give the sequences of three major Lys-C fragments of the isolate. The third and fifth columns give the sequences of two Glu-C fragments.

The positioning of the fragments shown in Fig. 7 are based on the apparent overlap in the various fragments. Based on this positioning, the most likely sequence for the protein is:
(H_2N)-Ala-Lys-Tyr-Asn-Lys-Ile-Lys-Ser-Arg-Gly-Ile-Lys-Ala-Asn-Thr-Phe-Lys-Lys-Leu-His-Asn-Leu-Ser-Phe-Leu-Tyr-Leu-Asp-His-Asn-Ala-Leu-Glu-Ser-Val-Pro-Leu-Asn-Leu-Pro-Glu-Ser-Leu-Arg-Val-Ile-His-Leu-Gln-Phe-Asn-Asn-Ile-Thr-Ser-Ile-Thr-Asp-Asp-Thr-Phe-Cys-Lys-Ala-Asn-Asp-Thr-Ser-Tyr-Ile-Arg-Asp-Arg-Ile-Glu-Glu-Ile-Arg-Leu-Glu-Gly-Asn-Pro-Val-Ile-Leu-Gly-Lys-His-Pro-Asn-Ser-Phe-Ile-Cys-Leu-Lys-Arg-Leu-Pro-Ile-Gly-Ser-Tyr-Ile-Asp-(COOH),

It will be appreciated that this sequence is not conclusive due to the nature of the analysis technique. Accordingly, the sequences given above may not be entirely accurate. The sequence may be confirmed by identifying the gene for the protein, sequencing the gene and deducing the amino acid sequence therefrom.

Amino acid composition analyses of the isolated deglycosylated protein were carried out with and without performic acid oxidation (performic acid oxidation permits detection of cysteic acid residues). The results of these analyses are indicated below.

Amino Acid	Without Performic Oxidation Integer Res/Mol	With Performic Oxidation Integer Res/Mol
Asx[1]	11	12
Thr	4	4
Ser	6	7
Glx[2]	8	7
Gly	5	8
Ala	4	4
Val	4	3
Ile	6	6
Leu	9	9
Tyr	3	ND
Phe	3	3
Lys	7	6
His	3	3
Arg	5	5
Pro	5	4
Met	0	0
Cys	ND	2
TOTAL	*83	83(86[3])

[1]Includes both Asp and Asn
[2]Includes both Glu and Gln

ND = not determined

[3]Includes three Tyr residues
*based on SDS-PAGE determined molecular weight of approximately 9600

To determine the amino acid composition of the

protein, the protein was hydrolyzed for 24 hr at 110°C in 6 N HCl containing 0.1% phenol. To determine the presence of Cys residues, performic acid oxidation was done prior to hydrolysis. Amino acids were detected on a Beckman 6300 analyzer using ninhydrin detection. It should be understood that these compositions are only an approximation due to the limitations of the analytical technique. Further it is clear that the molecular weight determined by SDS-PAGE does not conform with the molecular weight projected from the amino acid sequence analyses and is substantially lower than that projection. The invention provides the osteogenic protein in substantially pure form in which it is essentially free of other molecules with which it is associated in nature. In this regard, the term "substantially pure" intends a composition containing less than about 30% by weight contaminating protein, preferably less than about 10% contaminating protein, and most preferably less than about 5% by weight contaminating protein. The term "substantially pure" is used relative to proteins with which the osteogenic protein is associated in nature and is not intended to exclude compositions in which the osteogenic protein is admixed with nonproteinaceous pharmaceutical carriers or vehicles or proteinaceous pharmaceutical carriers or vehicles. The invention also provides the osteogenic protein in novel partially glycosylated or totally deglycosylated form (both of which are referred to herein as "deglycosylated"). The term "osteogenic" intends the ability to induce new bone formation either alone or in combination with a co-factor. Assays for osteogenic activity are described in the examples, infra.

Further characterization of the osteogenic protein of this invention may be carried out using procedures known in the art. Its isoelectric focusing pattern, isoelectric point, susceptibility to degradation by proteases or other chemicals such as acids or bases, and affinity to other materials such as other lectins, and the like may be so determined.

Based on the above amino acid sequence, oligonucleotide probes which contain the codons for a portion or all of the determined amino acid sequence are prepared and used to screen DNA libraries for genes encoding the osteogenic protein and substantially homologous genes that encode related proteins having osteogenic activity. The homologous genes may be from other species of mammals or animals (e.g., avians) or may represent other members of a family of related genes. The basic strategies for preparing oligonucleotide probes and DNA libraries, as well as their screening by nucleic acid hybridization, are well known to those of ordinary skill in the art. See, e.g., DNA CLONING: VOLUME I (D.M. Glover ed. 1985); NUCLEIC ACID HYBRIDIZATION (B.D. Hames and S.J. Higgins eds. 1985); OLIGONUCLEOTIDE SYNTHESIS (M.J. Gate ed. 1984); T. Maniatis, E.F. Frisch & J. Sambrook, MOLECULAR CLONING: A LABORATORY MANUAL (1982).

First, a DNA library is prepared. Since the identified protein is bovine, it is logical to probe a bovine library first, find full length clones and use the full length bovine clones to probe libraries of other mammalian species to identify the osteogenic protein gene (and thus the amino acid sequences) of other species. The library can consist of a genomic DNA library. Bovine and human genomic libraries are known in the art. See, e.g., Lawn et al., Cell (1978) 15:1157-1174. DNA libraries can also be constructed of cDNA prepared from a poly-A RNA (mRNA) fraction by reverse transcription. See, e.g., U.S. Patent Nos. 4,446,235; 4,440,859; 4,433,140; 4,431,740; 4,370,417; 4,363,877. The mRNA is isolated from an appropriate cell line or tissue that expresses the factor. Libraries from cells involved in bone formation (e.g., osteoblasts) or from osteotumors (e.g., osteosarcoma lines) are likely sources to probe for the osteogenic protein nucleic acids. Representative examples of such cell lines are the human amniotic line WISH (ATCC CCL25), the human osteosarcoma lines TE-85 (ATCC CRL1547) and MG63 (ATCC CRL1427), the human prostate carcinoma line PC-3 (ATCC CRL1435), the rat osteosarcoma line UMR-106 (ATCC CRL1661) and the mouse osteosarcoma line DUNN (available from Dr. Vital Ghanta, University of Alabama Medical School). cDNA (or genomic DNA) is cloned into a vector suitable for construction of a library. A preferred vector is a bacteriophage vector, such as phage lambda. The construction of an appropriate library is within the skill of the art.

Once the library is constructed, oligonucleotides to probe the library are prepared and used to isolate the desired osteogenic protein genes. The oligonucleotides are synthesized by any appropriate method. The particular nucleotide sequences selected are chosen so as to correspond to the codons encoding the known amino acid sequences of the osteogenic protein. Since the genetic code is redundant, it will often be necessary to synthesize several oligonucleotides to cover all, or a reasonable number, of the possible nucleotide sequences which encode a particular region of a protein. Thus, it is generally preferred in selecting a region upon which to base the probes, that the region not contain amino acids whose codons are highly degenerate. It may not be necessary, however, to prepare probes containing codons that are rare in the mammal from which the library was prepared. In certain circumstances, one of skill in the art may find it desirable to prepare probes that are fairly long, and/or encompass regions of the amino acid sequence which would have a high degree of redundancy in corresponding nucleic acid sequences, particularly if this lengthy and/or redundant region is highly characteristic of the protein. Probes covering the complete gene, or a substantial part of the genome, may also be appropriate, depending upon the expected degree of homology. Such would be the case, for example, if a cDNA of a bovine osteogenic protein was used to screen a human gene library for the corresponding human osteogenic protein. It may also be desirable to use two probes (or sets of probes), each to different regions of the gene, in a single hybridization experiment. Automated oligonucleotide synthesis has made the preparation of large families of probes relatively

straightforward. While the exact length of the probe employed is not critical, generally it is recognized in the art that probes from about 14 to about 20 base pairs are usually effective. Longer probes of about 25 to about 60 base pairs are also used.

The selected oligonucleotide probes are labeled with a marker, such as a radionucleotide or biotin using standard procedures. The labeled set of probes is then used in the screening step, which consists of allowing the single-stranded probe to hybridize to isolated ssDNA from the library, according to standard techniques. Either stringent or permissive hybridization conditions could be appropriate, depending upon several factors, such as the length of the probe and whether the probe is derived from the same species as the library, or an evolutionarily close or distant species. The selection of the appropriate conditions is within the skill of the art. See generally, NUCLEIC ACID HYBRIDIZATION, supra. The basic requirement is that hybridization conditions be of sufficient stringency so that selective hybridization occurs; i.e., hybridization is due to a sufficient degree of nucleic acid homology (e.g., at least about 75%), as opposed to nonspecific binding. Once a clone from the screened library has been identified by positive hybridization, it can be confirmed by restriction enzyme analysis and DNA sequencing that the particular library insert contains a gene for the osteogenic protein.

Alternatively, a DNA coding sequence for an osteogenic protein can be prepared synthetically from overlapping oligonucleotides whose sequence contains codons for the amino acid sequence of the protein. Such oligonucleotides are prepared by standard methods and assembled into a complete coding sequence. See, e.g., Edge, Nature (1981) 292:756; Nambair et al., Science (1984) 223:1299; Jay et al., J Biol Chem (1984) 259:6311.

Accordingly recombinant polynucleotides that encode the osteogenic polypeptides may be prepared and isolated by one or more of the above described techniques. The term "recombinant polynucleotide" as used herein denotes a polynucleotide of genomic, cDNA, semisynthetic or synthetic origin which, by virtue of its origin or manipulation (1) is not associated with all or a portion of the nucleic acid with which it is associated in nature or in the form of a library (2) is linked to a polynucleotide to which it is not linked in nature or (3) is not found in nature.

Recombinant DNA molecules containing the coding sequence for the osteogenic protein can be cloned in any suitable vector and thereby maintained in a composition substantially free of vectors that do not contain the coding sequence of the osteogenic protein (e.g., other library clones). Numerous cloning vectors are known to those of skill in the art, and the selection of an appropriate cloning vector is a matter of choice. Examples of recombinant DNA vectors for cloning and the host cells which they transform include bacteriophage lambda (E. coli), pBR322 (E. coli), pACYC177 (E. coli), pKT230 (gram-negative bacteria), pGV1106 (gram-negative bacteria), pLAFR1 (gram-negative bacteria), pME290 (non-E. coli gram-negative bacteria), pHV14 (E. coli and Bacillus subtilis), pBD9 (Bacillus), pIJ61 (Streptomyces), pUC6 (Streptomyces), actinophage C31 (Streptomyces), YIp5 (yeast), YCp19 (yeast), and bovine papilloma virus (mammalian cells). See generally, DNA CLONING: VOLUMES I & II, supra; MOLECULAR CLONING: A LABORATORY MANUAL, supra.

In one embodiment of the present invention, the coding sequence for an osteogenic protein gene is placed under the control of a promoter, ribosome binding site (for bacterial expression) and, optionally, an operator (collectively referred to herein as "control" sequences), so that the DNA sequence encoding the osteogenic protein (referred to herein as the "coding" sequence) is transcribed into RNA in the host cell transformed by the vector. The coding sequence may or may not contain a signal peptide or leader sequence. The determination of the point at which the mature protein begins and the signal peptide ends is easily determined from the N-terminal amino acid sequence of the mature protein. The osteogenic protein can also be expressed in the form of a fusion protein, wherein a heterologous amino acid sequence is expressed at the N-terminal. See e.g., U.S. Patents Nos. 4,431,739; 4,425,437.

The recombinant vector is constructed so that the osteogenic protein coding sequence is located in the vector with the appropriate control sequences, the positioning and orientation of the osteogenic protein coding sequence with respect to the control sequences being such that the coding sequence is transcribed under the control of the control sequences (i.e., by RNA polymerase which attaches to the DNA molecule at the control sequences). The control sequences may be ligated to the coding sequence prior to insertion into a vector, such as the cloning vectors described above. Alternatively, the coding sequence can be cloned directly into an expression vector which already contains the control sequence and an appropriate restriction site downstream from control sequences. For expression of the osteogenic protein coding sequence in procaryotes and yeast, the control sequences will be heterologous to the coding sequence. If the selected host cell is a mammalian cell, the control sequences can be heterologous or homologous to the osteogenic protein coding sequence, and the coding sequence can be genomic DNA, cDNA or synthetic DNA. Either genomic or cDNA coding sequence may be expressed in yeast. If glycosylation similar to the native molecule is desired, the gene may be expressed in yeast or mammalian cells (COS, CHO, or CV-1 cells) using vectors and procedures known in the art. In this regard, initial tests at selected concentrations indicate tentatively that the totally deglycosylated protein is not active. For this reason, expression in eukaryotes that are capable of effecting glycosylation may be essential to make an active protein by recombinant procedures.

A number of procaryotic expression vectors are known in the art. See, e.g., U.S. Patent Nos. 4,440,859; 4,436,815; 4,431,740; 4,431,739; 4,428,941; 4,425,437; 4,418,149; 4,411,994; 4,366,246; 4,342,832. See also British Patent Specifi-

cations GB 2,121,054; GB 2,008,123; GB 2,007,675; and European Patent Specification 103,395. Yeast expression vectors are known in the art. See, e.g., U.S. Patent Nos. 4,446,235; 4,443,539; 4,430,428. See also European Patent Specifications 103,409; 100,561; and 96,491.

Recombinant osteogenic protein can be produced by growing host cells transformed by the expression vector described above under conditions whereby the osteogenic protein is produced. The osteogenic protein is then isolated from the host cells and purified. If the expression system secretes osteogenic protein into growth media, the protein can be purified directly from cell-free media. If the recombinant protein is not secreted, it is isolated from cell lysates. The selection of the appropriate growth conditions and recovery methods are within the skill of the art or are apparent from the recovery methods used to isolate the native osteogenic proteins. The recombinant protein may be recovered by affinity chromatography using the antibodies produced in accordance with the invention. Recombinant osteogenic protein may be unglycosylated or have a different glycosylation pattern than the native molecule depending upon the host that is used to produce it. As indicated above, the deglycosylated protein may not be active. It would be useful, however, for making antibodies that recognize sequential epitopes of the protein.

Either native, deglycosylated, or synthetic (recombinant) osteogenic protein can be used to produce antibodies, both polyclonal and monoclonal. The term "antibody" is intended to include whole Ig of any isotype or species as well as antigen binding fragments and chimeric constructs. If polyclonal antibodies are desired, purified osteogenic protein is used to immunize a selected mammal (e.g., mouse, rabbit, goat, horse, etc.) and serum from the immunized animal later collected and treated according to known procedures. Compositions containing polyclonal antibodies to a variety of antigens in addition to the osteogenic protein can be made substantially free of antibodies which are not anti-osteogenic protein antibodies by passing the composition through a column to which osteogenic protein has been bound. After washing, polyclonal antibodies to the osteogenic protein are eluted from the column. Monoclonal anti-osteogenic protein antibodies can also be readily produced by one skilled in the art. The general methodology for making monoclonal antibodies by hybridomas is well known. Immortal, antibody-producing cell lines can also be created by techniques other than fusion, such as direct transformation of B lymphocytes with oncogenic DNA, or transfection with Epstein-Barr virus. See, e.g., M. Schreier et al., HYBRIDOMA TECHNIQUES (1980); Hammerling et al., MONOCLONAL ANTIBODIES AND T-CELL HYBRIDOMAS (1981); Kennett et al., MONOCLONAL ANTIBODIES (1980).

By employing osteogenic protein (native, deglycosylated or synthetic) as an antigen in the immunization of the source of the B-cells immortalized for the production of monoclonal antibodies, a panel of monoclonal antibodies recognizing epitopes at different sites on the osteogenic protein molecule can be obtained. Antibodies which recognize an epitope in the binding region of the protein can be readily identified in competition assays between antibodies and protein. Antibodies which recognize a site on the osteogenic protein are useful, for example, in the purification of the protein from cell lysates or fermentation media, in characterization of the protein and in identifying immunologically related proteins. Such immunologically related proteins (i.e., that exhibit common epitopes with the osteogenic protein) are another aspect of the invention. In general, as is known in the art, the antiosteogenic protein antibody is fixed (immobilized) to a solid support, such as a column or latex beads, contacted with a solution containing the osteogenic protein, and separated from the solution. The osteogenic protein, bound to the immobilized antibodies, is then eluted.

Osteogenic Compositions

The osteogenic protein of the invention may be used to induce de novo bone formation in circumstances where bone is not normally formed. The protein may thus be used prophylactically to reduce the likelihood of fracture, improve fixation of artificial joints, repair congenital or trauma-induced bone defects, or in cosmetic plastic surgery. The protein may also be used to enhance bone formation in instances where bone is normally formed, such as in fracture repair, replacement of surgically removed bone, or repair of bone damaged by periodontal disease or in other tooth or alveolar ridge repair processes. In such uses, the protein will be administered locally, such as by implantation, at the desired site of bone formation.

The protein may also be administered systemically, such as intravenously, to treat indications associated with insufficient bone formation and/or undesirable levels of bone resorption such as localized, regionalized or generalized osteoporosis or to stimulate bone marrow progenitor cells in the treatment of malfunctions or dysfunctions of the hematopoietic system such as chronic and acute mylocytic leukemia and other cancers of the hematopoietic system or in post-irradiation treatment to stimulate bone marrow stem cells to divide and differentiate. In this regard TGF-beta may be used pre-irradiation to suppress marrow stem cell reproduction and differentiation and the invention protein may be used post-irradiation to stimulate such cells.

Initial tests of the osteogenic protein composition indicate that it is necessary, in the concentrations and formulations tested, to coadminister a protein having TGF-beta activity to achieve bone induction at nonbony sites. It may be that TGF-beta induces proliferation of bone forming cells and the osteogenic protein of the invention induces differentiation of such cells. In this regard, TGF-beta (TGF-beta1, TGF-beta2, other members of the TGF-beta family, or mixtures thereof) may enhance the process of bone induction through ancillary activities such as antiinflammatory activity, chemotactic activity, and the like. Other molecules that exhibit such activities may also be useful as co-factors for

bone-induction. The protein may, of course, be active at other concentrations or in other formulations. Further it may not be necessary to coadminister TGF-beta at bony sites since the amount of endogenous TGF-beta present at the site of action or in systemic applications may be sufficient.

The osteogenic protein of the invention will normally be formulated in osteogenically effective amounts with pharmaceutically acceptable solid or fluid carriers, for local injection or implantation at the desired site of activity or systemic administration by conventional parenteral routes. Preferably the formulations for local administration include a matrix material that is capable of presenting the protein at the desired site of activity as well as providing a structure for developing bone and cartilage. Potential matrices may be biodegradable or nonbiodegradable and be chemically or biologically defined. Examples of such materials are calcium sulfate, hydroxyapatite, tricalciumphosphate, polyorthoesters, polylactic-polyglycolic acid copolymers, collagen, bioglass, and the like. Formulations for systemic administration will typically involve liquid vehicles that are commonly used for parenteral administration of proteinaceous therapeutics.

The osteogenic protein of the invention may be conjugated with other molecules to increase its water-solubility, increase its half-live, or enhance its ability to bind to bone. For instance, it may be conjugated to polyethylene glycol to increase its water solubility or to bone-binding molecules such as bisphosphonates (e.g. 1-hydroxyethylidene-1,1-bisphosphonic acid, dichloromethylene bisphosphonic acid, and 3-amino-1-hydroxypropylidene-1,1-bisphosphonic acid) and fluorochromes (e.g. tetracyclines, calcein blue, xylenol orange, calcein green, and alizarin complexone red) to target the protein to bony sites. Various agents for conjugating molecules to proteins are well known in the art and include aldehydes, carbodiimides, and other bifunctional moieties.

The amount of osteogenic protein administered may vary depending upon the carrier used, the patient (age, sex, medical history, species) and the site and condition being treated. For local implantation, the weight ratio of osteogenic protein to carrier in the formulation will typically be in the range of about 1:5,000 to 1:50,000. The weight ratio of osteogenic protein to TGF-beta in the composition will usually be in the range of 10:1 to 1:10. The implant may be placed at a predetermined site in the patient by conventional surgical techniques, such as implantation or injection.

For systemic administration the amount of osteogenic protein will usually range between 30 g/kg body weight and 1 mg/kg body weight. TGF-beta may be added to the systemic formulations, if necessary, in the above proportions. In addition it may be desirable to combine the osteogenic protein with other therapeutics, such as, for instance in the case of osteoporosis, fluoride, calcitonin, vitamin D metabolites, and parathyroid hormone. Because the protein is nonspecies specific in its activity it may be used to treat mammals in general including sport, pet, and farm animals and humans.

Examples

The following is intended to illustrate the process for purification of native osteogenic protein as applied to a particular sample and the osteogenic activity of the isolated protein. It is not intended to limit the invention.

A. Preparation of Demineralized Bone

Bovine metatarsal bone was obtained fresh from the slaughterhouse and transported on ice. Bones were cleaned of all periosteum and marrow with high pressure water, crushed into fragments using a liquid-nitrogen-cooled grinder and pulverized into powder using a liquid-nitrogen-cooled mill. The pulverized bone was washed four times for 20 minutes in 4°C deionized water (8 liters/kg). The bone was then washed overnight with the same volume of deionized water at 4°C. The bone powder was demineralized for 5 hr in 0.5 N HCl (21 liter/kg) at 4°C. The acid was decanted, and the demineralized bone powder was washed several times with 4°C deionized water until the wash reached a pH > 3. The excess water was removed on a suction filter.

B. Extraction of Noncollagenous Proteins

Demineralized bone powder was extracted with 4 M guanidine-HCl, 10 mM EDTA pH 6.8 (2 liters/kg bone powder) for 16 hr at 4°C. The suspension was suction-filtered to recover the guanidine-HCl-soluble fraction and concentrated at least 5-fold by ultrafiltration using a 10,000 dalton cut-off membrane (S10Y10 Amicon spiral cartridge).

C. Gel Filtration

The extract from ©B, redissolved in 4 M guanidine-HCl, was fractionated on a Sephacryl S-200 column equilibrated in 4 M guanidine-HCl, 0.02% sodium azide, 10 mM EDTA, pH 6.8. Fractions were assayed by their absorbance at 280 nm and the fractions were combined as shown in Figure 2. The fraction indicated by <----> in Figure 2 constitutes a low molecular weight (LMW, 10,000-30,000 daltons) protein fraction possessing the greatest activity. This fraction was pooled and dialyzed against 6 changes of 180 volumes of deionized water and lyophilized. All operations except lyophilization and dialysis (4°C) were conducted at room temperature.

D. Ion Exchange Chromatography

The pooled fraction from ©C was dissolved in 6 M urea, 10 mM NaCl, 1 mM NEM, 50 mM sodium acetate, pH 4.8 and centrifuged at 10,000 rpm for 5 min. The supernatant was fractionated on a CM52 (a commercially available CMC) column equilibrated in the same buffer. Bound proteins were eluted from the column using a 10 mM to 400 mM NaCl gradient in the same buffer, and a total volume of 350 ml at a flow rate of 27 ml/hr. Proteins eluted with 10-150 mM NaCl (the <----> of Figure 3) were collected and dialyzed against 6 changes of 110 volumes of deionized water for 4 days and lyophilized. All of the foregoing operations were conducted at room

temperature except dialysis (4°C).

E. ConA Chromatography

The fraction obtained in step D above was enriched in osteogenic activity by affinity chromatography using concanavalin A (ConA)-Sepharose 4B (Pharmacia). In order to minimize leaching of ConA from the column during chromatography, the resin was cross-linked with glutaraldehyde essentially as described by K.P. Campbell, D.H. MacLennan, J Biol Chem (1981) 256:4626. Briefly, resin was pelleted (500 x g, 5 min) and washed twice with 4 volumes of 250 mM $NaHCO_3$, pH 8.8. The resin was then equilibrated in the same buffer for 6-8 hrs at 4°C. After pelleting, the resin was cross-linked by the addition of 4 volumes of 250 mM $NaHCO_3$, pH 8.8, 250 mM methyl-alpha-D-mannopyranoside (alpha-MM), 0.03% glutaraldehyde with gentle mixing for 1 hr at room temperature. The reaction was quenched by washing the resin twice in 1 M Tris-HCl, pH 7.8. The resin was stored in the same buffer containing 0.01% Thimersol at 4°C until use.

Samples for ConA chromatography were solubilized in 1% deoxycholate at pH 8.0. Any small amount of precipitate was removed by centrifugation 12,000 x g, 5 minutes.

Prior to chromatography, cross-linked resin was first equilibrated with >5 column volumes of 50 mM Tris, pH 8.0 followed by >5 column volumes of 1% sodium deoxycholate. Samples were loaded and nonbound fractions collected by washing with 1% DOC. Elution was monitored by OD_{280} Bound material was eluted with 0.5 M alpha-MM in 1% DOC as shown in Figure 4.

F. Chromatography on Heparin-Sepharose

The bound fraction eluted from the ConA column was reequilibrated by chromatography on a GH-25 column (Pharmacia) equilibrated in 6 M urea, 0.1 M NaCl, 50 mM Tris-HCl pH 7.2 heparin-sepharose buffer. Approximately 80 mg (1 mg/ml) were loaded on a 25 ml large heparin sepharose column (Pharmacia). The column was washed of all unbound material. Then bound proteins were eluted with the same equilibrating buffer but containing 0.5 M NaCl as shown in Figure 5. About 5-8 mg of heparin-sepharose bound proteins were recovered.

G. Chromatography on C18-RP-HPLC

The pH of the heparin-bound fraction was lowered below 5 by adding TFA. Final purification of the heparin-bound fraction was achieved using reversed phase HPLC. The columns used were a Vydac TP-RP18 4.6 mm x 25 cm and 1.0 x 25 cm. Solvent A was 0.1% aqueous trifluoroacetic acid (TFA) and B 90% acetonitrile in A. Bound proteins were eluted from the column with a 32-62% B solvent gradient at a rate of 1%/min. The osteogenic protein composition eluted between 47-50% solvent B as shown in Figure 6. 140-200 ug protein were recovered. Amino acid composition and amino acid sequences of the protein were determined using standard procedures and are described above and shown in Figure 7.

H. Deglycosylation

Glycopeptidase F cleaves N-linked oligosaccharides at the innermost N-acetylglucosamine residue. High mannose, hybrid and complex oligosaccharides are susceptible to the enzyme. Osteogenic protein was iodinated by the chloramine-T method. Labeled protein was digested for 12-15 hours with 6.7 units/ml glycopeptidase F (Boehringer Mannheim) in 0.1 M Tris-HCl, pH 7.4, 10 mM EDTA, at 37°C.

Both the glycosylated and deglycosylated forms were analyzed by sodium dodecyl sulfate/15% polyacrylamide slab gels prepared according to standard methods. Figure 8 is a photograph of the autoradiograph.

I. In vivo Bioassay of Materials

The osteoinductive activity of the protein composition described above was evaluated in vivo as follows.

I.1. Formulation of Osteogenic Protein Composition

The protein was used as a 24 ug/ml solution in 1% TFA, approximately 45% acetonitrile. TGF-beta2 was used as a 30 ug/ml solution in 1% TFA, approximately 45% acetonitrile.

One ml of osteogenic protein solution and 1.4 ml of the TGF-beta solution were stirred with 9 ml of Vitrogen® collagen-in-solution (Collagen Corporation, Palo Alto, CA) at 4°C for 5 min. 243 mg of porous hydroxylapatite/tricalciumphosphate ceramic in particulate form (Zimmer Corp., Warsaw, IN) was added and the mixture was incubated at 4°C for 5 min and then lyophilized. This mixture provided sufficient material for 6 implants. A like formulation was made lacking TGF-beta.

I.2. Implantation

The ability of the formulations of I.1 to induce endochondral bone formation in rats was determined as follows. Portions of the lyophilized formulations were [hydrated with approximately one volume of water, allowed to soak for 5 min and molded into approximately 5 x 5 mm bodies for implantation. The implants contained ~4 ug/implant of osteogenic protein and 0 or ~7 ug/implant of TGF-beta. The implants were surgically placed in 34-40 day old male Sprague-Dawley rats on either side of the ventral thoracic region. Explants were removed at 14 days and evaluated biochemically for bone formation.

I.3. Assay for Alkaline Phosphatase

The level of alkaline phosphatase (AP) activity in the explants is a measure of osteogenic activity. To determine alkaline phosphatase (AP), the explants were cut in small pieces and homogenized in 10 volumes (1/10) of ice-cold 1.5 M NaCl, 3 mM $NaHCO_3$, pH 7.5 The homogenized samples were then centrifuged at 12,000 rpm for 50 min at 4°C, and an aliquot of the supernatant was diluted 1:10 in cold, distilled water. The method of Huggins, et al., J EXP Med (1961) 114:761, was used to assess alkaline phosphatase using polystyrene plates.

I.4. Results

Figure 9 is a bar graph showing the results of the AP assay. As indicated the formulation containing no TGF-beta (a) was essentially non-active in the assay; whereas the formulation with TGF-beta (b) showed substantial activity. Histological examination of the explants confirmed the assay results.

Similar tests were carried out using osteogenic protein compositions that had been reduced and alkylated or trypsinized. These tests indicate that the protein is deactivated by such treatments.

J. In vitro Assay of Materials

J.1. Effect on Rat Osteosarcoma Cell Line (ROS 17/2.8)

Cells were seeded in multiwell plates at 2500 cells/cm^2. After overnight attachment in Hams F12 medium, 10% fetal bovine serum, the cells were incubated with varying amounts of the osteogenic protein composition for 72 hours in serum-free Hams F12 medium. The control was serum-free Hams F12 medium containing no osteogenic protein. After incubation the cells were rinsed twice with PBS and lysed with 0.1% Triton. Aliquots were taken and tested for AP activity using p-nitrophenylphosphate (PNP) as a substrate. Figure 10 shows the results of those assays. As shown, the osteogenic protein produced a significant increase in AP activity which was dose-dependent at concentrations of 3-30 ng/ml.

J.2. Effect on Adult Baboon Explant Bone Cells

Cancellous bone was obtained from adult baboon long bone by biopsy. The bone was minced and cellular outgrowth followed for up to three weeks. Cells were trypsined and used immediately or replated. Replated cells were used within the first three passages. Tests for AP activity were carried out as in J.1. above. The results of those tests were reported in the table below.

Osteogenic Protein Concentration (ng/ml)	AP specific activity (nmPNP/ug protein/min)
100	6.0×10^{-3}
50	9.1×10^{-3}
25	4.8×10^{-3}
12.5	3.5×10^{-3}
Control	3.0×10^{-3}

Similar tests were carried out on the human osteoblastic cell line MG-63 and on rat primary osteoblastic cells. However, no increase in AP activity was observed.

J.3. Effect on Multinucleated Cell (MNC) Formation

Multinucleated cells are "osteoclast-like" cells. Osteoclasts are involved in bone resorption. Inhibition of the formation of osteoclasts is believed to limit bone resorption.

Bone marrow mononuclear cells from normal human donors were isolated using Hypaque-Ficoll (Histopaque-1077) gradient centrifugation. Mononuclear cells were cultured in alpha-MEM containing 20% horse serum at 10^6 cells/ml in 24-well plates ($5x10^5$ cells/well). All cultures were maintained in a humidified atmosphere of 4% CO_2-air at 37°C. Cultures were fed weekly by removing half of the medium and replacing an equal volume of fresh medium. Osteogenic protein was added to the cultures in the presence of 1,25-dihydroxyvitamin D (D_3), 10^{-8}M. After designated periods of culture (usually 3 weeks) cells were fixed with 5% glutaraldehyde and stained with Wright's stain. Cells containing 3 or more nuclei were counted as MNCs using an inverted phase microscope. Figure 11 is a graph showing the results of these tests. As shown, the osteogenic protein caused a dose-dependent inhibition of MNC formation at the concentrations tested.

K. Production and Testing of Antibodies to the Osteogenic Protein

K.1. Production of Polyclonal Antibodies

Polyclonal antibodies to (1) a synthetic 30 mer polypeptide having a sequence corresponding to the amino acids 1-30 of Figure 7 except for a Leu ----> Asn substitution at position 25 and (2) the native protein purified from bone as described above were prepared and characterized as follows.

Antiserum to the 1-30 mer was raised in a rabbit by injecting the rabbit with 500 ug of the polypeptide in complete Freund's adjuvant (CFA), followed by boosts of 500 ug of the polypeptide in incomplete Freund's adjuvant (ICFA) at approximately three week intervals. The antiserum was obtained after the fourth boost and had a titer as measured by ELISA of $>1:10,000$. Rabbit antiserum to the native protein was raised similarly using an initial injection of 50 ug protein in CFA followed by boosts of 50 ug protein in ICFA. This antiserum had a titer of $>1:10,000$ by ELISA.

The antiserum to the 1-30 mer was tested in Western blots on the purified native osteogenic protein, deglycosylated native osteogenic protein, and on crude native osteogenic protein (Con-A bound material), all fixed post-blotting with 0.2% glutaraldehyde. The antiserum detected the purified native osteogenic protein at ≥ 1 ug and also recognized the deglycosylated protein and the crude protein. The antiserum to the native osteogenic protein recognized the native protein at ≥ 100 ng in Western blots.

K.2. Production of Monoclonal Antibodies

Murine monoclonal antibodies to the purified native osteogenic protein were prepared as follows. From two fusions 25 positive wells were identified. A group of female Balb/c mice was injected intraperitoneally (IP) with 10-20 ug of purified native osteogenic protein in CFA. The animals were boosted with 10-20 ug of protein in ICFA. Following the third boost, the mice were bled and serum antibody titers against the protein checked by ELISA. Two animals were found to have titers of $\geq 1:40,000$. They were given a final intravenous (IV) injection of 20 ug protein four days prior to the fusion.

Fusion to the SP2/0 myeloma (GM3659 B, NIGMS Human Genetic Mutant Cell Repository, Camden, NJ) was performed essentially according to the protocol of Oi and Herzenberg, "Immunoglobulin-producing Hybrid Cell Lines" in Selected Methods in Cellular Immunology, Mishell and Shiigi, eds., W.H. Freeman and Co., San Francisco, pp. 357-362, (1980). Spleen cells from the animals were mixed with SP2/0 at a ratio of 5:1. 50% polyethylene glycol 1500 (Boehringer-Mannheim Biochemicals, Indianapolis, IN) was used as the fusagen. Cells were plated at 10^6 cells/well along with resident peritoneal cells at 4×10^3 cells/well in DMEM with high glucose (4.5 g/l) supplemented with 20% FCS (Hyclone Laboratories, Logan, UT), 2 mM L-glutamine, 2 mM sodium pyruvate, nonessential amino acids, penicillin and streptomycin. In this procedure, aminopterin was replaced by azaserine (Sigma) according to the procedure by Larrick et al., Proc Natl Acad Sci USA (1983) 80:6376, and added along with thymidine and hypoxanthine on day 1 after the fusion.

From two fusions 25 positive wells were identified.

All 25 were positive for immunoprecipitation of ^{125}I-labeled osteogenic protein and 23 of the 25 were positive in an ELISA against the protein. The supernatant from one uncloned well (3B2.17, previously designated FO13-3B2) was particularly positive and was used in a Western blot. In this testing synthetic peptides corresponding to amino acid segments 1-30, 62-95 and 76-105 of the osteogenic protein sequence were made and 1-2 ug of each was applied to separate lanes in the gel. Blots were probed with 50-100 ug/ml of purified antibody. This antibody recognized $\geqq$300 ng protein as well as deglycosylated protein. The antibody also picked up the protein in a crude fraction (total Con-A bound) and was found to recognize the C-terminal peptide (76-105) but not the N-terminal peptide (1-30). Another clone, designated 2C11.6, was found to recognize the internal 62-95 segment. Clones 3B2.17 and 2C11.6 were subcloned by limiting dilution and were found to be stable and to be IgG isotype. These clones are being deposited in the American Type Culture Collection (ATCC) under the provisions of the Budapest Treaty.

The antibodies produced by the two clones were testing for their ability to neutralize or block the activity of the osteogenic protein. ROS or BMS-2 cells were cultured with 30 ng/ml of the osteogenic protein in the presence or absence of antibody at varying concentrations. Both of these cell lines normally exhibit substantial increases in AP when cultured in media containing osteogenic protein at 30 ng/ml. The antibody from 3B2.17 was found to neutralize this effect of the osteogenic protein at concentrations >10 ug/ml.

Modifications of the above-described modes of carrying out the invention that are obvious to those of skill in the arts relevant to the invention are intended to be within the scope of the following claims.

Claims

1. A substantially pure polypeptide having osteogenic activity and an internal sequence in the N-terminal portion as follows
-Lys-Tyr-Asn-Lys-Ile-Lys-Ser-Arg-Gly-Ile-Lys-Ala-Asn-Thr-Phe-Lys-Lys-Leu-His-Asn-Leu-Ser-Phe-Leu-Tyr-Leu-Asp-His-Asn-Ala-Leu-Glu-
and substantially pure polypeptides that are substantially equivalent and substantially homologous thereto.

2. The polypeptide of claim 1 wherein the amino acid immediately preceding the initial Lys of the internal sequence is Ala and defines the amino terminal of the polypeptide.

3. A polypeptide having an internal sequence in the N-terminal portion of the polypeptide as follows:
-Lys-Tyr-Asn-Lys-Ile-Lys-Ser-Arg-Gly-Ile-Lys-Ala-Asn-Thr-Phe-Lys-Lys-Leu-His-Asn-Leu-Ser-Phe-Leu-Tyr-Leu-Asp-His-Asn-Ala-Leu-Glu-
wherein said polypeptide is deglycosylated relative to a native osteogenically active polypeptide having said sequence and deglycosylated polypeptides that are substantially equivalent and substantially homologous thereto.

4. A substantially pure osteogenically active polypeptide having the following amino acid sequence:
(H_2N)-Ala-Lys-Tyr-Asn-Lys-Ile-Lys-Ser-Arg-Gly-Ile-Lys-Ala-Asn-Thr-Phe-Lys-Lys-Leu-His-Asn-Leu-Ser-Phe-Leu-Tyr-Leu-Asp-His-Asn-Ala-Leu-Glu-Ser-Val-Pro-Leu-Asn-Leu-Pro-Glu-Ser-Leu-Arg-Val-Ile-His-Leu-Gln-Phe-Asn-Asn-Ile-Thr-Ser-Ile-Thr-As p-Asp-Thr-Phe-Cys-Lys-Ala-Asn-Asp-Thr-Ser-Tyr-Ile-Arg-Asp-Arg-Ile-Glu-Glu-Ile-Arg-Leu-Glu-Gly-Asn-Pro-Val-Ile-Leu-Gly-Lys-His-Pro-Asn-Ser-Phe-Ile-Cys-Leu-Lys-Arg-Leu-Pro-Ile-Gly-Ser-Tyr-Ile-Asp-(COOH),
and substantially pure polypeptides that are substantially equivalent and substantially homologous thereto.

5. A polypeptide having the following amino acid sequence:
(H_2N)-Ala-Lys-Tyr-Asn-Lys-Ile-Lys-Ser-Arg-Gly-Ile-Lys-Ala-Asn-Thr-Phe-Lys-Lys-Leu-His-Asn-Leu-Ser-Phe-Leu-Tyr-Leu-Asp-His-Asn-Ala-Leu-Glu-Ser-Val-Pro-Leu-Asn-Leu-Pro-Glu-Ser-Leu-Arg-Val-Ile-His-Leu-Gln-Phe-Asn-Asn-Ile-Thr-Ser-Ile-Thr-As p-Asp-Thr-Phe-Cys-Lys-Ala-Asn-Asp-Thr-Ser-Tyr-Ile-Arg-Asp-Arg-Ile-Glu-Glu-Ile-Arg-Leu-Glu-Gly-Asn-Pro-Val-Ile-Leu-Gly-Lys-His-Pro-Asn-Ser-Phe-Ile-Cys-Leu-Lys-Arg-Leu-Pro-Ile-Gly-Ser-Tyr-Ile-Asp-(COOH),
wherein said polypeptide is deglycosylated relative to a native osteogenically active polypeptide having said sequence and deglycosylated polypeptides that are substantially equivalent and substantially homologous thereto.

6. A composition for inducing bone formation and/or inhibiting bone resorption comprising an effective amount of an osteogenically active polypeptide of claim 1, 2 or 4.

7. The composition of claim 6 which further includes an effective amount of TGF-beta.

8. A method of inducing bone formation in vivo at a predetermined site in a living mammal comprising placing the composition of claim 6 or 7 at said site.

9. A method of inducing bone marrow cell production in a living mammal comprising administering an effective amount of the composition of claim 6 or 7 to the mammal systemically.

10. A method of treating osteoporosis in a living mammal comprising administering an effective amount of the composition of claim 6 or 7 to the mammal systemically.

11. In the method of treating a living mammal for a cancer of the hematopoietic system comprising irradiating the mammal to kill neoplastic hematopoietic cells, the improvement comprising administering a sufficient amount of the composition of claim 6 or 7 to the mammal systemically after said irradiation to stimulate hematopoietic stem cell division.

12. The method of claim 11 wherein a sufficient amount of TGF-beta is administered systemically to the mammal prior to said irradiation to suppress hematopoietic stem cell division.

13. Antibody that binds to a polypeptide of claim 1, 2, 3, 4 or 5.

14. A recombinant polynucleotide encoding a polypeptide of claim 1, 2, 3, 4 or 5.

15. A recombinant vector containing a recombinant polynucleotide of claim 14 and capable of directing the expression of the polypeptide encoded thereby.

16. A recombinant host cell or microorganism containing the recombinant vector of claim 15 and capable of permitting expression of said polypeptide.

17. A process for producing a polypeptide of claim 1, 2, 3, 4 or 5 comprising culturing the recombinant host cell or microorganism of claim 16.

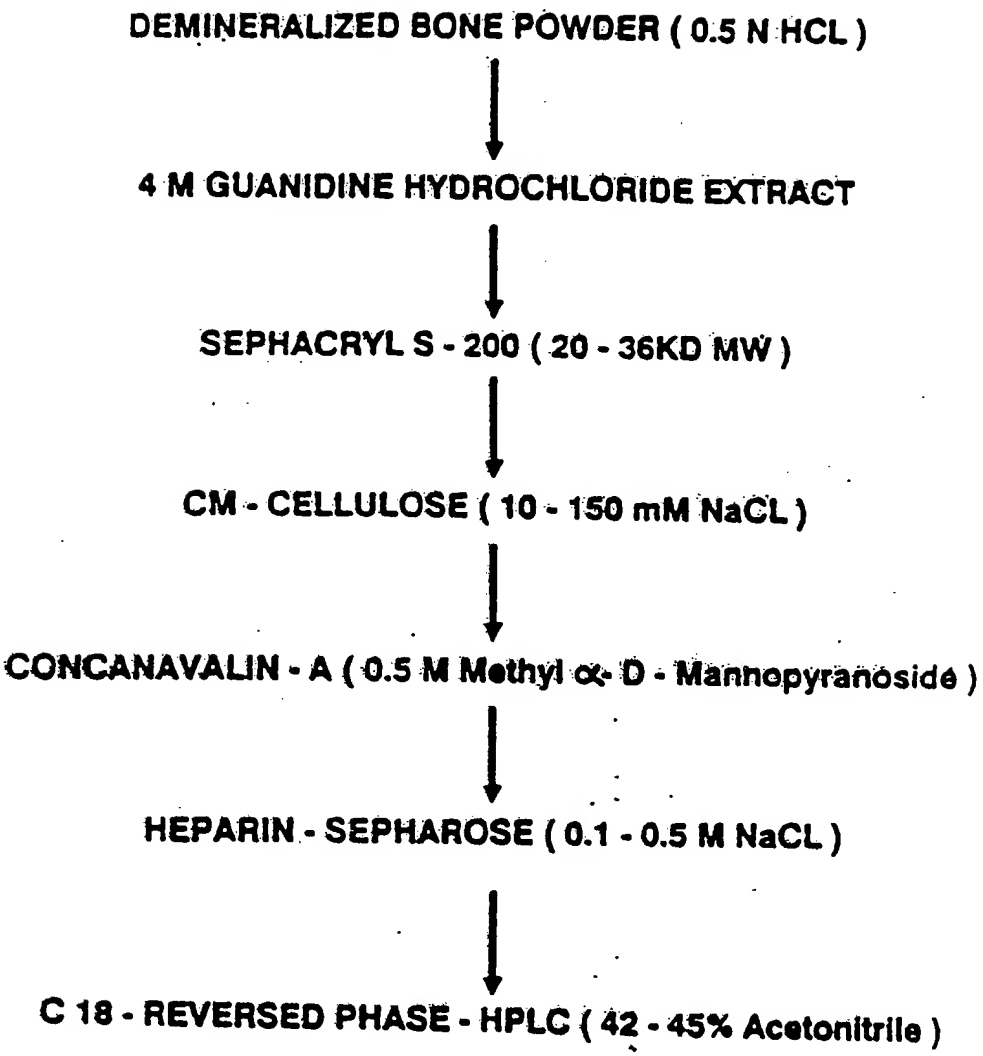

DEMINERALIZED BONE POWDER (0.5 N HCL)
4 M GUANIDINE HYDROCHLORIDE EXTRACT
SEPHACRYL S - 200 (20 - 36KD MW)
CM - CELLULOSE (10 - 150 mM NaCL)
CONCANAVALIN - A (0.5 M Methyl α- D - Mannopyranoside)
HEPARIN - SEPHAROSE (0.1 - 0.5 M NaCL)
C 18 - REVERSED PHASE - HPLC (42 - 45% Acetonitrile)

FIGURE 1

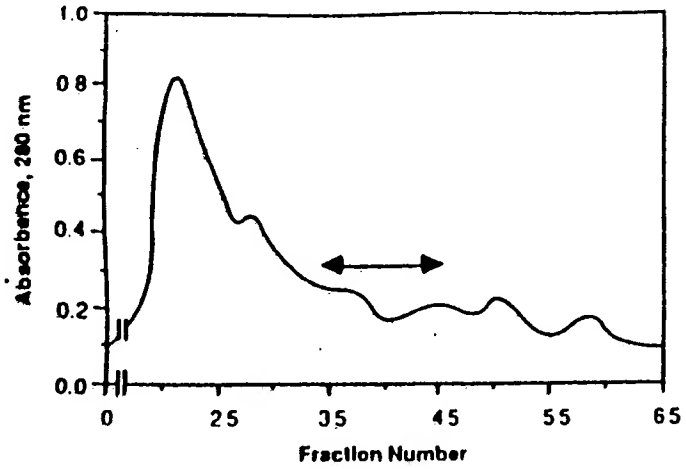


FIGURE 2

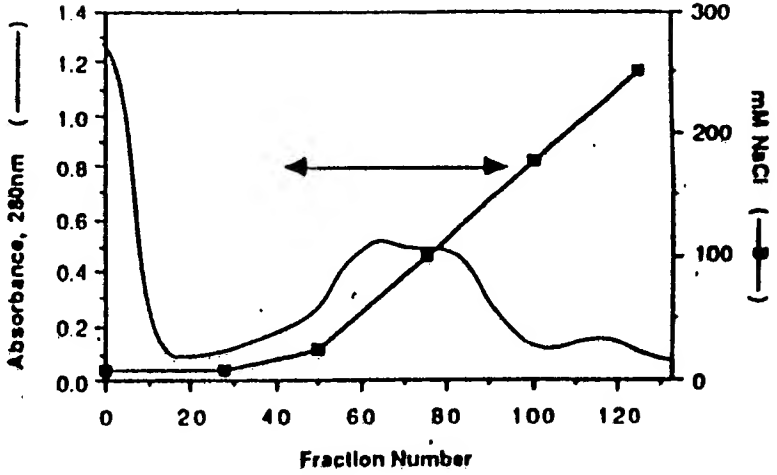
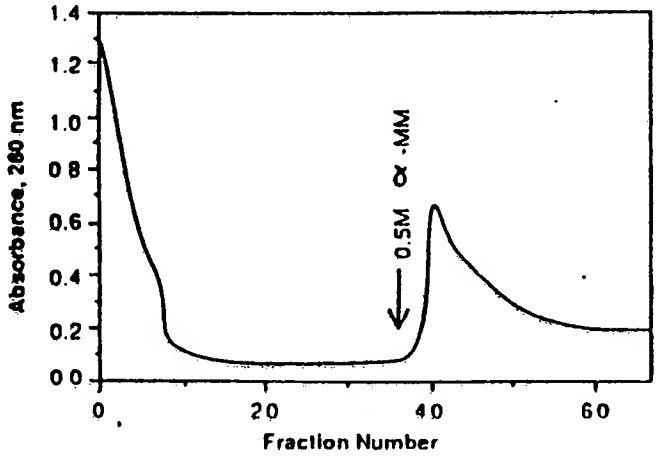


FIGURE 3



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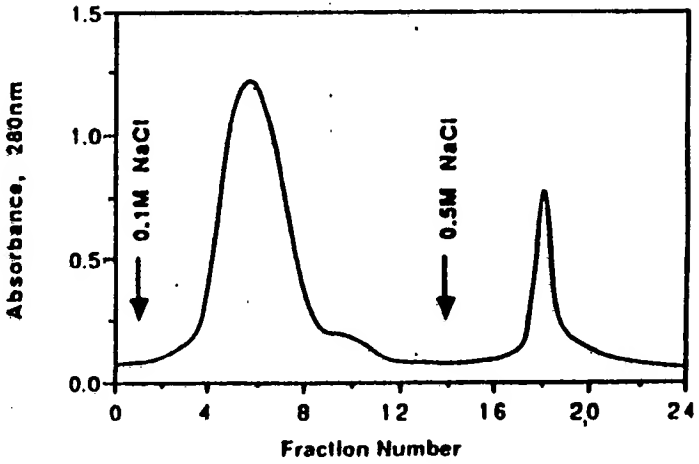

FIGURE 5

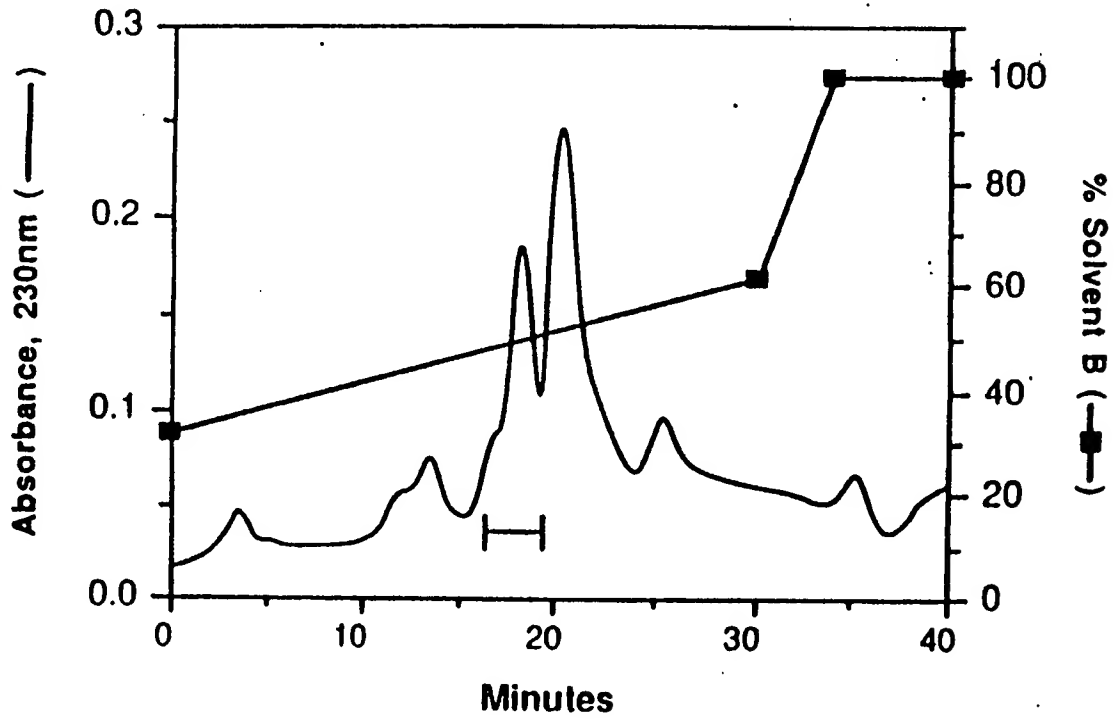
0.3
0.2
0.1
0.0
Absorbance, 230nm (——)
0
10
20
30
40
Minutes
100
80
60
40
20
0
% Solvent B (—■—)

FIGURE 6

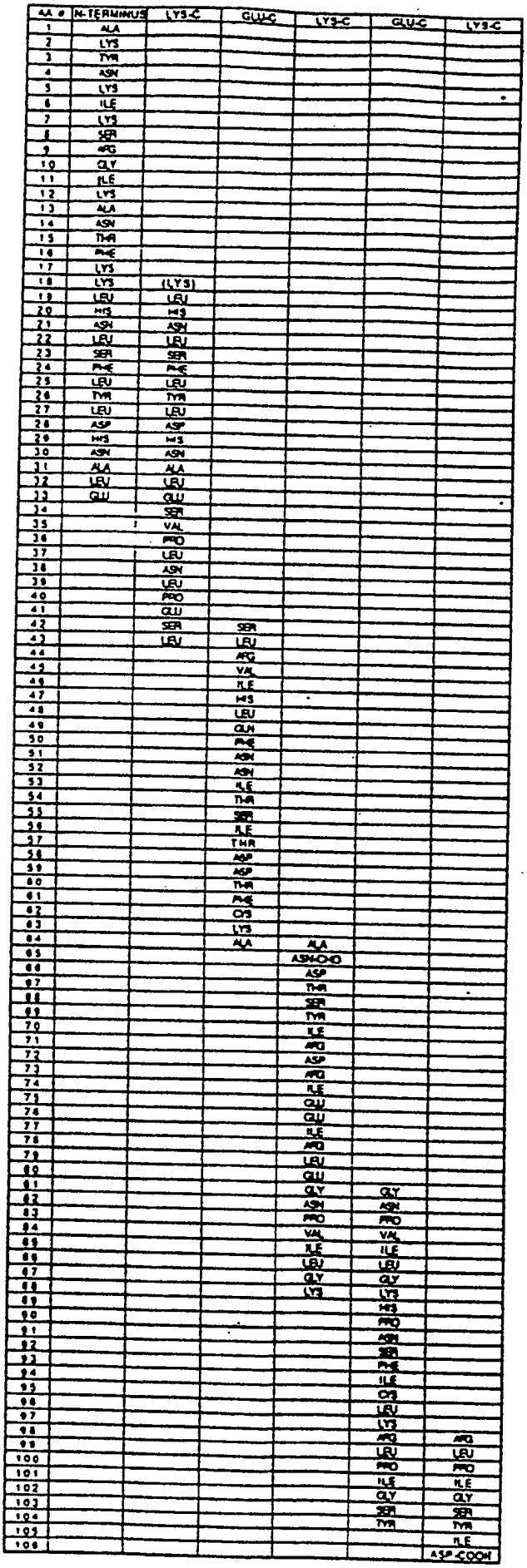

FIGURE 7

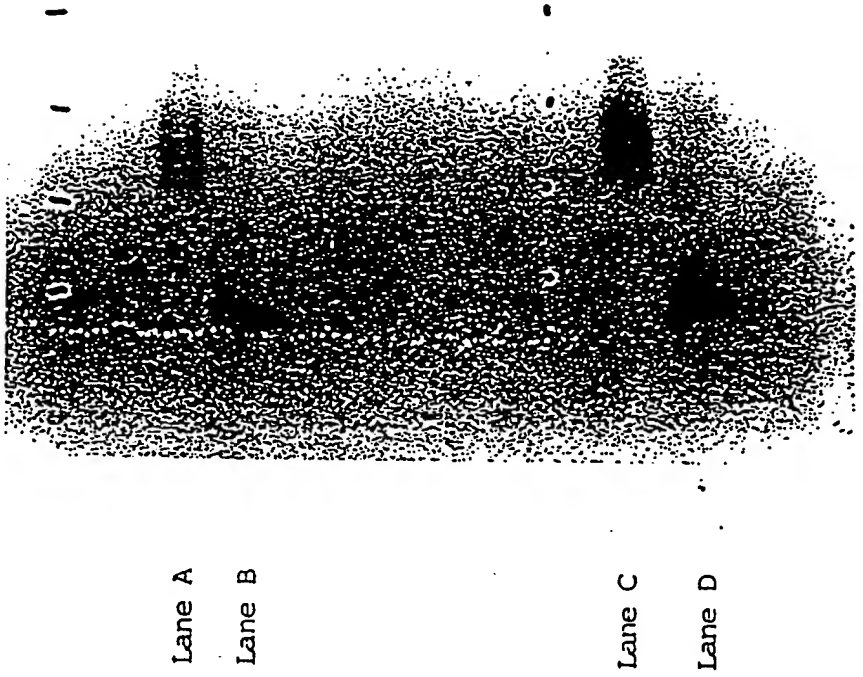

FIGURE 8

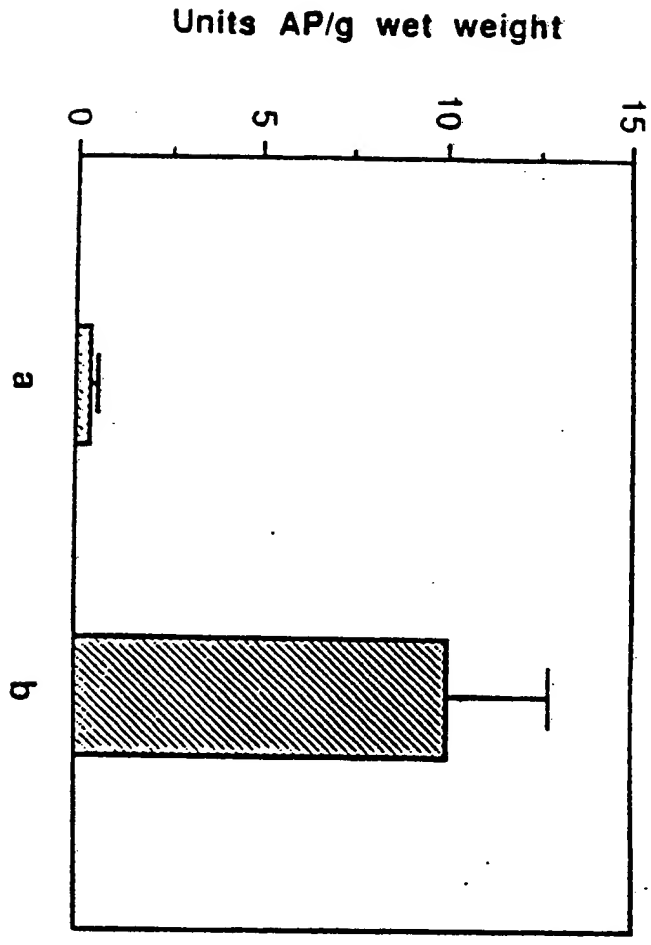

FIGURE 9

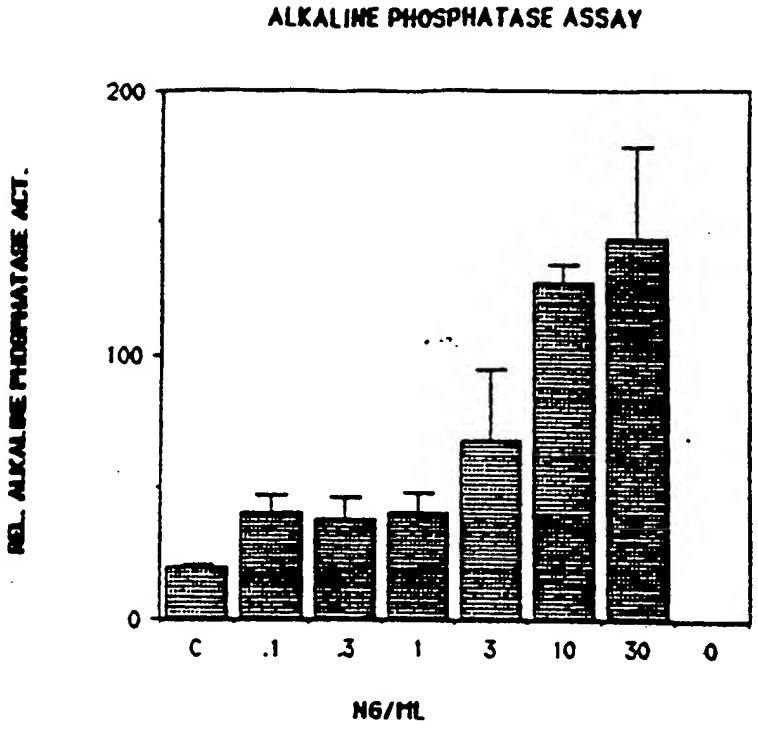
ALKALINE PHOSPHATASE ASSAY
REL. ALKALINE PHOSPHATASE ACT.
200
100
0
C
.1
.3
1
3
10
30
0
NG/ML

FIGURE 10

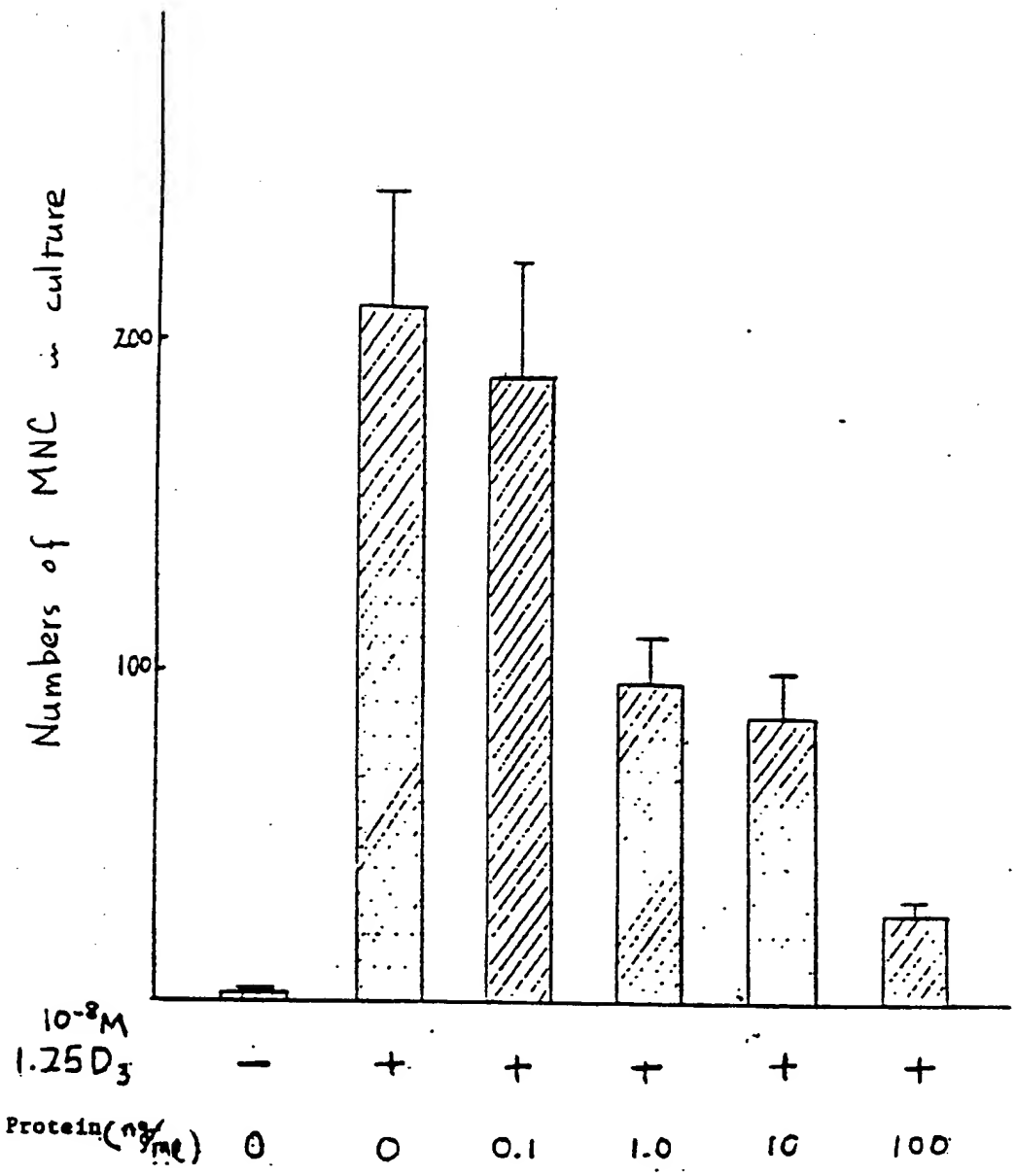
Numbers of MNC in culture
200
100
10^{-8}M
1.25D3
Protein(ng/ml)
0
0
0.1
1.0
10
100

FIGURE 11

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415,555 4 October 1989 (04.10.89) US

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(54) Title: OSTEOGENIC FACTORS

(57) Abstract

The present invention provides an osteogenically active protein preparation characterized by a molecular weight of from about 31,000 to 34,000 daltons as characterized by non-reducing sodium dodecyl sulfate polyacrylamide gel electrophoresis and by the characteristic of eluting from a reverse phase high performance liquid chromatography column equilibrated with buffers containing trifluoroacetic acid and acetonitrile by eluting within the concentrations of 35 % to 45 % acetonitrile. The invention further provides improved methods for isolating such preparations and genes encoding all or a portion of polypeptide subunits of dimers comprising the osteogenic protein preparation.

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OSTEOGENIC FACTORS

This is a continuation-in-part of application Serial No. 256,034 filed October 11, 1988.

BACKGROUND OF THE INVENTION

The present invention relates to novel preparations of osteogenic factors, methods for their isolation and uses thereof (to repair bone defects). The preparations so isolated exhibit the ability to promote or stimulate the formation of bone at the site of their application. Bone is a highly specialized connective tissue with unique mechanical properties derived from its extensive matrix structure. A network of fibrous bundles composed of the protein, collagen, is presumed to provide the tension-resistant behavior of bone. In addition, other materials including proteoglycans, noncollagenous proteins, lipids and acidic proteins associated with a mineral phase consisting primarily of poorly crystallized hydroxyapatite are deposited in the extensive matrix architecture of bone. Bone tissue is continuously renewed, by a process referred to as remodeling, throughout the life of mammals. This physiologic process might serve to maintain the properties of a young tissue.

The processes of bone formation and renewal are carried out by specialized cells. Osteogenesis vis-a-vis morphogenesis and growth of bone is presumably

carried out by the "osteoblasts" (bone-forming cells). Remodeling of bone is apparently brought about by an interplay between the activities of the bone-resorbing cells called "osteoclasts" and the bone-forming osteoblasts. The boney skeleton is thus not only an architectural structure with a mechanical function but also is a living tissue capable of growth, modeling, remodeling and repair. Since these processes are carried out by specialized living cells, chemical (pharmaceutical/hormonal), physical and physicochemical alterations can affect the quality, quantity and shaping of bone tissue.

A variety of pathological disorders as well as physical stress (for example, fracture) necessitate active formation of bone tissue at rates that are significantly higher than that which can be supported by the normal milieu of the body. It is thus of value to identify physiologically acceptable substances (hormones/pharmaceuticals/growth factors) that can induce the formation of bone at a predetermined site where such substances are applied, for example, by implantation. Such agents could either provide a permissive matrix structure for the deposition of bone-forming cells, or stimulate bone-forming cells, or induce the differentiation of appropriate progenitors of bone-forming cells.

The presence of proteinaceous and prostaglandin-like growth stimulators for osteoblasts has been examined, see reviews: Raisz, L.G., et al., The New England Journal of Medicine, Vol. 309, No. 1, pp. 29-35 (1983) and Raisz, L.G., et al., The New England Journal of Medicine, Vol. 309, No. 2, pp. 83-89 (1983).

The observation that a bone graft from the same individual or a compatible individual leads to the formation of new healthy bone at the site of the graft,

led to the hypothesis that bone contains active proteins which promote local osteogensis. Urist, et al. disclosed evidence that bone matrix-associated noncollagenous proteins can be isolated by dissociative treatment of demineralized bone powder and that this mixture of noncollagenous proteins contain the local osteoinductive capability which was designated by Urist (e.g., Science, Vol. 150, p. 893 (1965)) as bone morphogenetic activity.

A variety of osteogenic, cartilage-inducing and bone-inducing protein preparations have been described in the art. Urist, et al. and others have described various partially fractionated protein preparations with osteoinductive properties. These preparations are fractionated from the noncollagenous protein mixture extracted using different dissociative treatment of demineralized bone powder and subjecting the extract to various protein fractionation steps. Several such preparations have been characterized by different assays to determine their biological activities and by protein components identified using different standard protein analytical methods.

Urist, et al., Proceedings of The Society for Experimental Biology And Medicine, 162, pp. 48-53 (1979), disclosed isolation of bone morphogenetic protein (BMP) from demineralized rabbit bone matrix. The reference discloses that BMP appears to contain a multitude of major protein components having molecular weights in the range of between 94,000 daltons (94K) to less than 14,000 daltons (14K) based on reducing SDS polyacrylamide gel electrophoretic (SDS-PAGE) analysis.

Urist, et al. in Proc. Nat'l. Acad. Sci. USA, Vol. 76, No. 4, pp. 1828-1832 (April, 1979), disclosed another preparation of BMP obtained from demineralized rabbit bone matrix. Five protein fractions each characterized by having a major component with an

apparent molecular weight of 94K, 68K, 43K, 21K and 14.3K were identified by subjecting these preparations to SDS polyacrylamide gel electrophoresis. All five protein preparations were eluted from a gel column with α-methylmannoside. Four of the five preparations, namely those with major components of molecular weights ranging from 68K to 14.3K were eluted with ethyleneglycol and two preparations, namely those with major components of molecular weights from 21K and 14.3K were precipitated with calcium phosphate. All three groups of eluates were found to have comparable BMP activity. The reference suggests that the BMP activity in the third group (the preparations characterized by major components of 21K and 14.3K proteins) may result from dissociation of a low molecular weight hydrophobic molecule carried by a glycoprotein. The reference suggests the alternative possibilities that BMP could be a single glycoprotein molecule, that the biologic activity may be a function of a protein aggregate or that BMP activity may not be associated with bone glycoprotein at all (pg. 1831).

Urist, U.S. Patent No. 4,294,753, disclosed that the molecular weight of BMP may range between about 20K and 63K (col. 4, lines 45-61). The reference disclosed that BMP preparation isolated from rabbit dentin matrix protein mixture appears to have a major component with a molecular weight of about 23K. Because a protein fraction obtained from osteosarcoma cells has a molecular weight of 63K, it was suggested that the matrix free 63K protein may be a BMP precursor.

Hanamura and Urist, et al., Clin. Ortho. and Rel. Res., No. 153, pp. 232-240 (November-December, 1980), disclosed the purification of osteosarcoma produced material with bone morphogenetic activity into three main fractions characterized by having a major component of molecular weight of 16K, 12.5K and 7K,

respectively. Fractions characterized by a major component with a higher molecular weight including a 22K protein were observed during initial purification steps; active fractions purified from such preparations did not contain the 22K protein. Based on these results, the 12.5K and 16K proteins were tentatively identified as BMP.

Conover and Urist, et al., The Chemistry and Biology of Mineralized Connective Tissues, Elsevier North Holland, Inc., Arthur Veis, editor, pp. 597-606 (1981), discloses the isolation of a BMP fraction from demineralized rabbit dentin. Preparations containing proteins having average molecular weights of 30K, 23K, 18K, 15K and 12K were identified. While it was suggested that a 23K protein might represent the active BMP fraction, it was acknowledged that the active fraction might be the 18K, 15K or 12K proteins which they were unable to separate from the 30K and 23K fractions.

Farley, et al., Biochemistry, Vol. 21, No. 14, pp. 3502-3507 (1982), discloses purification of a skeletal growth factor from demineralized human bone matrix with an apparent molecular weight of 83K. The disclosure makes reference to a 1981 reference (Trans. Annu. Meet.-Orthop. Res. Soc., 6, 136 (1981)) by Urist, Conover and others, describing bone morphogenetic protein as having a molecular weight of 23K.

Urist, et al., Clin. Ortho. and Rel. Res., No. 162, pp. 219-232, discloses a low molecular weight bone morphogenetic protein fraction extracted from bovine bone matrix and fractionated by ion exchange and gel chromatography. The reference discloses that bovine BMP may consist of components ranging in molecular weight from 12K to 30K with the main components corresponding to molecular weights of 23K, 18K and 12K. The reference suggests that the 18K component is

the active protein of the group because of its invariable presence in active fractions.

Urist, et al., Proc. Soc. Exp. Biol. and Med., 173, pp. 194-199 (1983), identifies human bone morphogenetic protein (hBMP) extracted from demineralized human bone matrix as an 18K molecular weight protein. The 18K protein was identified as putative hBMP as a result of its invariable presence in chromatographic fractions having high hBMP activity and general absence in those fractions lacking such activity. 34K, 24K and 14K protein components isolated from the demineralized bone were found not to induce bone formation.

Seyedin, et al., U.S. Patent Nos. 4,434,094, and 4,627,982 describe the work in Urist, U.S. Patent No. 4,294,753 and state that in the Urist patent, BMP was not fully characterized. The Seyedin patents describe a process for partially purifying an osteogenic factor and describe the factors as having a molecular weight of less than or equal to 30K.

Urist, et al., Science, 220, pp. 680-686 (1983), again identifies BMP purified from demineralized bone matrix as an 18K molecular weight protein. Variable quantities of 14K, 24K and 34K proteins were isolated with the 18K protein but the reference discloses that each of the last three protein fractions can be removed without loss of BMP activity. The reference states that the 18K fraction is responsible for BMP activity and suggests that the 34K, 24K and 14K proteins are individually inactive but are subunits of a larger BMP complex with the 18K protein.

Urist, et al., Proc. Nat'l. Acad. Sci. USA, 81, pp. 371-375 (1984), confirms that bovine BMP has an apparent molecular weight of 18.5K daltons. The publication further discloses other bone derived proteins with apparent molecular weights of 17.5K and

17K, proteins with higher molecular weights of 34K, 24K and 22K and a protein with a lower molecular weight of 14K. The publication provided the N-terminal sequence for the 17.5K protein which had an unblocked amino terminus.

Urist, European Patent Application No. 212,474, discloses peptide fragments having molecular weights between about 4K and 7K comprising at least an active portion of the osteoinductive and immunoreactive domain of the 17.5K BMP molecule.

Wang, et al., Patent Cooperation Treaty Application No. WO 88/00205, claiming priority based on applications including U.S. Serial No. 880,776 filed July 1, 1986, discloses a bovine bone inductive factor which is isolated from demineralized bone powder by a procedure comprising a number of chromatographic and dialysis steps. The bone inductive factor so isolated was found to contain, as judged by a non-reducing SDS-PAGE analysis, one or more proteins having a molecular weight of approximately 28,000 to 30,000 daltons. Reducing SDS-PAGE analysis of the active protein(s) yielded two major bands having the mobility of proteins having molecular weights of 18,000 daltons and 20,000 daltons respectively. Wang, et al. discloses three bovine proteins designated BMP-1, BMP-2 and BMP-3 where BMP is bone morphogenetic protein and provides peptide sequences for the proteins. Wang, et al. also discloses the nucleotide sequences and amino acid sequences predicted thereby of four human proteins designated BMP-1, BMP-2 Class I, BMP-2 Class II and BMP-3.

Wozney, et al., Science 242, pp. 1528-1533 (1988), describes the nucleotide sequences and amino acid sequences predicted thereby of three human complementary DNA clones (designated BMP-1, BMP-2A and BMP-3) corresponding to three polypeptides present in an

extract of bovine bone which is capable of inducing de novo bone formation. Recombinant human BMP-1, BMP-2A and BMP-3 proteins were said to be independently capable of inducing the formation of cartilage in vivo. The nucleotide sequence and derived amino acid sequence of a fourth complementary DNA clone (designated BMP-2B) is also described. The BMP-1, BMP-2A, BMP-2B and BMP-3 proteins of this publication appear to correspond, respectively, to the BMP-1, BMP-2 Class I, BMP-2 Class II and BMP-3 proteins, respectively, of Wang, et al.

Sen, U.S. Patent No. 4,804,744 issued February 14, 1989, discloses a preparation of an osteogenic protein which is a member of the P3 family of proteins and which has an apparent molecular weight of 22,000 to 24,000 daltons as revealed by coomassie blue staining of reducing SDS-PAGE analysis.

Lyons, et al., Proc. Nat'l. Acad. Sci. USA 86, pp. 4554-4558 (1989), describes the nucleotide sequence and derived amino acid sequence of a complementary DNA clone (designated Vgr-1) encoding a mouse protein which contains homologous regions for the deduced amino acid sequences of BMP-2A, BMP-2B and BMP-3.

Luyten, et al., J. Biol. Chem. 264, pp. 13377-13380 (1989), describes the purification and partial amino acid sequence analysis of a polypeptide present in an extract of bovine bone said to be capable of inducing de novo bone formation. This protein, designated osteogenin, has an apparent molecular mass of 22,000 daltons as judged by reducing SDS-PAGE analysis, and an apparent molecular mass of 30,000 to 40,000 daltons as judged by a non-reducing SDS-PAGE analysis. The amino acid sequences reported for osteogenin are said to show considerable homology to BMP-3 as described by Wozney, et al.

Bentz, et al., J. Bone and Mineral Res., 4 Supplement 1, p. S280 No. 650 (1989) and Bentz, et al., J. Cell Biol., 107, 162a No. 918 (1989) describe a protein material isolated from demineralized bone matrix said to promote osteoinduction in the rat. The osteoinductive factor (OIF) was identified as a glycoprotein and was said to exhibit osteoinductive activity only in the presence of TGF-β1 or TGF-β2. OIF had an apparent molecular mass of 22,000 to 28,000 daltons based on SDS gel electrophoresis and was identified as a monomeric molecule in light of the fact that reduction does not alter its mobility on SDS-PAGE.

SUMMARY OF THE INVENTION

The present invention is directed to mammalian bone matrix-derived proteins which exhibit the ability to promote or stimulate local osteogenesis (bone formation) at sites of implantation in mammals. Specifically, the invention provides preparations of osteogenic proteins and involves extraction and purification of such osteogenically active protein preparations including extraction of bone matrix proteins under dissociative (denaturing) conditions followed by further purification using one or more methods such as specific elution of these proteins from gel filtration chromatographic columns, ion-exchange chromatographic columns, metal chelate affinity columns, hydrophobic adsorption chromatographic columns and reverse phase HPLC (high performance liquid chromatography) columns using an acetonitrile gradient. These preparations obtained using such purification procedures are clearly characterized by their respective chromatographic behaviors using these gel filtration, ion-exchange, metal chelate, hydrophobic adsorption and reverse phase HPLC columns as well as by their ability to induce local bone formation in animals

at a predetermined site where they are applied either alone or in admixture with a suitable pharmaceutically acceptable carrier. The invention further provides methods of inducing bone formation in a mammal comprising administering to the mammal effective amounts of the osteogenic preparation. Also provided are pharmaceutically acceptable compositions comprised of one or more of the proteins or active polypeptides in conjunction with a physiologically acceptable matrix material. The invention further provides polypeptide subunits of the osteogenically active 31,000 to 34,000 dalton protein molecules, designated P3 OF 31-34, which are found associated with the P3 proteins of bone nucleotide sequences encoding certain of the subunits of P3 OF 31-34 or portions thereof and novel osteogenically active heterodimer proteins comprising certain of these subunits.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 represents the elution profile obtained by Sepharose CL-6B column chromatography of the proteins obtained in an eight hour extraction of demineralized calf bone powder with 4 M GuHCl-0.01 M Tris·HCl buffer (pH 7.0).

Figure 2 represents the elution profile obtained by Sephacryl S-200 column chromatography, in 4 M GuHCl-0.01 M Tris·HCl buffer (pH 7.0), of the proteins contained in the active fraction obtained from Sepharose CL-6B column chromatography.

Figure 3 represents the elution profile of proteins present in the active pool from Sephacryl S-200 column chromatography on a reverse phase Protesil 300 octyl column using an acetonitrile gradient for the elution of proteins.

Figure 4 represents the results of electrophoretic analysis of purified bone matrix

proteins on discontinuous sodium dodecyl sulfate-polyacrylamide gels in the presence of a reducing agent followed by coomassie blue staining to detect the protein material.

Figure 5A represents the elution profile obtained by high performance liquid chromatography, on a reverse phase C8 column, of fragments of porcine P3 protein; the fragments were generated by the enzymatic digestion of porcine P3 protein using Staphylococcus aureus V8 protease.

Figure 5B represents the elution profile obtained by high performance liquid chromatography, on a reverse phase C8 column, of fragments of bovine P3 protein; the fragments were generated by the enzymatic digestion of bovine P3 protein using Staphylococcus aureus V8 protease.

Figure 6A represents the elution profile obtained by high performance liquid chromatography, on a reverse phase C18 column, of fragments of porcine P3 protein; the fragments were generated by the enzymatic digestion of reduced, carboxymethylated porcine P3 protein using Staphylococcus aureus V8 protease.

Figure 6B represents the elution profile obtained by high performance liquid chromatography, on a reverse phase C18 column, of fragments of human P3 protein; the fragments were generated by the enzymatic digestion of reduced, carboxymethylated human P3 protein using Staphylococcus aureus V8 protease.

Figure 7 represents the results of competitive radioimmunoassays measuring the ability of radiolabelled test antigen to bind to specific antibody molecules in the presence of competing unlabelled antigen preparations.

Figure 8 represents the elution profile obtained by Sephacryl S-200 column chromatography, in 4 M GuHCl-0.01 M Tris·HCl buffer (pH 7.0), of the proteins contained in the active 5K-100K fraction.

Figure 9 illustrates an alternative method for the purification of P3 OF 31-34 (osteogenic factors) proteins from calf bone.

Figure 10A shows the apparent molecular weight of the osteogenic factors as determined by non-reducing SDS polyacrylamide gel electrophoresis followed by silver staining.

Figure 10B shows reducing SDS polyacrylamide gel electrophoresis of P3 OF 31-34 proteins followed by silver staining.

Figure 11A shows the isolation of the subunits of the P3 OF 31-34 proteins (osteogenic factors) by reverse phase HPLC.

Figure 11B shows the apparent molecular weights of the subunits as detected by silver staining of reducing SDS polyacrylamide gel electrophoretic analysis.

Figure 12 represents the alignment of the amino terminal and internal sequences of subunits A, B, C and D of the P3 OF 31-34 proteins with homologous regions from the deduced amino acid sequences of cDNA clones encoding hOD and hOE isolated according to the invention and the polypeptides designated in the literature as BMP-2A, BMP-2B and Vgr-1.

Figure 13A represents the elution profile obtained by high performance liquid chromatography, on a reverse phase C18 column, of the PS Pool.

Figure 13B shows non-reducing SDS polyacrylamide gel electrophoresis of P3 OF 31-34 proteins eluting in fractions 26, 27 and 28 from the reverse phase HPLC of the PS Pool.

Figure 14A shows the isolation and identification of subunits of the P3 OF 31-34 proteins eluting in fraction 26 from the reverse phase HPLC of the PS Pool.

Figure 14B shows the isolation and identification of subunits of the P3 OF 31-34 proteins eluting in fraction 28 from the reverse phase HPLC of the PS Pool.

Figure 15 shows reducing SDS polyacrylamide gel electrophoresis of P3 and P3 OF 31-34 proteins isolated and visualized using either Coomassie stain, or autoradiography following Western Blot analysis utilizing antibodies generated against synthetic peptides of the N terminal sequences of subunit A (AbANt) and subunit D (AbDNt).

Figure 16 shows reducing SDS polyacrylamide gel electrophoresis of reduced subunits A and D before and after treatment with either endo H or N-glycanase.

Figure 17 shows the nucleotide and derived amino acid sequences of PCR-amplified DNA from U-2 OS mRNA, designated hOD.

Figure 18 shows the nucleotide and derived amino acid sequences of PCR-amplified DNA from U-2 OS mRNA, designated hOE.

Figure 19A shows the homology between the derived amino acid sequences of the PCR-amplified sequences designated hOD and hOE.

Figure 19B shows the homology between the nucleotide sequences of the PCR-amplified sequences designated hOD and hOE.

DETAILED DESCRIPTION OF THE INVENTION

Using certain chromatographic procedures known in the art, each of several proteins have been purified starting from crude protein extracts of demineralized bone powder. As judged by the migration of these proteins in polyacrylamide gels under reducing conditions, using the procedure essentially as described in Laemmli, U.K., Nature, Vol. 227, pp. 680-685 (1970), different protein species have been assigned numbers

such as P1, P2 and the like in the order of decreasing apparent molecular weight. Equivalent proteins have been obtained from bones of different mammals. The osteogenically active polypeptides of the invention have the characteristic that they copurify under certain purification procedures with a family of immunologically related P3 proteins, having an apparent molecular weight of 22,000 to 24,000 daltons. Similarly, a P3 protein isolated from human bone and purified according to the procedure essentially as described herein is immunologically related to the calf P3 protein and has an apparent molecular weight of 22,000 to 24,000 daltons revealed by coomassie blue staining of reducing SDS-PAGE analysis.

The osteogenically active preparation obtained using the method of this invention is sometimes referred to herein as the P3 protein. The invention further concerns the ability to obtain osteogenically active P3 proteins from bones of various mammals using the method of this invention. The osteogenically active protein preparations obtained from different mammalian bones using the method of this invention constitute members of a family of proteins, referred to herein as an immunologically related family of P3 proteins. The members of this family show substantial equivalence to each other with regard to characteristics such as (i) osteogenic activity, (ii) chromatographic characteristics in dissociative gel filtration columns, (iii) elution from hydrophobic reverse phase HPLC columns in acetonitrile, (iv) an essential homogeneity with regard to a molecular weight of between about 22,000 and 24,000 daltons revealed by coomassie blue staining of reducing SDS-PAGE analysis, (v) characteristics of certain major peptide fragments generated by proteolytic treatment and (vi) reactivity in an immunoassay directed toward certain immunogenic determinants characteristic in such preparations.

The invention further relates to the identification, in the P3 proteins, of proteins which, during gel filtration under non-reducing and dissociative conditions, elute as proteins having apparent molecular weights within the range of about 25,000 to 38,000 daltons, and more specifically, when analyzed by non-reducing SDS-PAGE followed by silver staining, migrate as proteins having apparent molecular weights within the range of about 31,000 to 34,000 daltons. These proteins are designated P3 OF 31-34, indicating osteogenically active 31,000 to 34,000 dalton protein molecules which are found associated with the P3 proteins of bone and are distinct from bone-derived protein molecules of similar molecular weight which lack osteogenic activity.

The invention further provides alternative protein fractionation methods of isolating these 31,000 to 34,000 dalton molecular weight protein constituents inherent in the P3 proteins which are characterized by the ability to promote osteogenesis. The P3 OF 31-34 osteogenic protein material yields four distinct peaks when analyzed by reverse phase HPLC after reduction. When analyzed by reducing SDS-PAGE and silver staining, three of the peaks are characterized as protein subunits migrating with apparent molecular weights within the range of 17,500 to 19,000 daltons, and the fourth peak is characterized as a protein subunit migrating with an apparent molecular weight within the range of 16,000 to 17,500 daltons.

Applicants have characterized the protein subunits of P3 OF 31-34 and designated them as subunits A, B, C and D. The subunits have been characterized by sequencing of various internal and presumptive amino-terminal polypeptide fragments. Applicants have utilized the polymerase chain reaction (PCR) technique to amplify sequences of human cDNA

homologous to that encoding subunit D and have provided amino acid and nucleotide sequences for human subunit D (hOD). Applicants have also identified a sequence of human cDNA encoding what is characterized as polypeptide subunit E (hOE) which may be a new osteogenic polypeptide or may correspond to the bovine subunit A polypeptide. Applicants have also determined that the P3 OF 31-34 osteogenic protein material is comprised of polypeptide dimers including a heterodimer of subunit D with subunit B and a heterodimer of subunit A (and/or subunit E) with subunit B. The P3 OF 31-34 osteogenic material may further comprise heterodimers of subunit A (and/or subunit E) with subunit C and heterodimers of subunit D with subunit C given the high degree of homology (80%) between subunits B and C.

The invention provides polypeptide subunit D of P3 OF 31-34 such as isolated from bovine bone and a purified and isolated nucleic acid from human DNA comprising a nucleotide sequence selected from the group consisting of a nucleotide sequence encoding subunit D of P3 OF 31-34, a nucleotide sequence which encodes the same sequence of amino acids making up subunit D of P3 OF 31-34, a nucleotide sequence which is homologous with 80% of the nucleotides encoding subunit D of P3 OF 31-34 and a nucleotide sequence which would be homologous with 80% of the nucleotides encoding subunit D of P3 OF 31-34 but for the redundancy of the genetic code. The invention also provides recombinant expression systems for subunit D including vectors including nucleic acid sequences encoding subunit D of P3 OF 31-34, a cell transformed therewith and a polypeptide expression product of such a transformed cell.

The invention further provides polypeptide subunit E of P3 OF 31-34 and a purified isolated nucleic acid comprising a nucleotide sequence selected from the group consisting of a nucleotide sequence encoding

subunit E of P3 OF 31-34, a nucleotide sequence which encodes the same sequence of amino acids making up subunit E of P3 OF 31-34, a nucleotide sequence which is homologous with 80% of the nucleotides encoding subunit E of P3 OF 31-34 and a nucleotide sequence which would be homologous with 80% of the nucleotides encoding subunit E of P3 OF 31-34 but for the redundancy of the genetic code. The invention also provides recombinant expression systems for subunit E including vectors including nucleic acid sequences encoding subunit E of P3 OF 31-34, a cell transformed therewith and a polypeptide expression product of such a transformed cell.

The invention also provides an osteogenic preparation comprising a dimer comprising subunit D of P3 OF 31-34 and, additionally, an osteogenic preparation comprising a heterodimer comprising subunits D and B of P3 OF 31-34 linked by at least one disulfide bond. The invention still further provides an osteogenic preparation comprising a dimer comprising subunit E of P3 OF 31-34 and, additionally, an osteogenic preparation comprising a heterodimer comprising subunits E and B of P3 OF 31-34 linked by at least one disulfide bond. The invention further comprises an osteogenic preparation comprising a dimer comprising subunit A of P3 OF 31-34 and, additionally, an osteogenic preparation comprising a heterodimer comprising subunits A and B of P3 OF 31-34 linked by at least one disulfide bond.

The osteogenic protein preparations, namely the P3 protein, a preparation containing the P3 OF 31-34 protein or a preparation containing subunits A, B, C, D or E or homo- or heterodimers thereof as described herein, may be used to form a composition for implantation into a mammal by admixture with a physiologically acceptable matrix material. In addition, devices for implantation into mammals

comprising a structural member encoated with the osteogenic factor/matrix composition are provided by the invention.

It may be possible, using procedures well known in the art, for example, chemical, enzymatic or recombinant DNA techniques, to obtain polypeptides derived from the osteogenic proteins described herein which exhibit the ability to promote or stimulate osteogenesis. For example, any of polypeptide subunits A, B, C, D and E or nucleic acid encoding such polypeptides or analogs not directly provided herein may be obtained according to procedures well known to those skilled in the art. Such procedures include obtaining the complete amino acid sequence of any of the polypeptide subunits and screening DNA libraries from one or more mammalian species with polynucleotide probes based thereon, and including identifying cells expressing any of the polypeptide subunits present in P3 OF 31-34 by using a labelled antibody or oligonucleotide according to the present invention, isolating mRNA therefrom and preparing cDNA from the isolated mRNA. The invention further provides a process for the preparation of an osteogenic protein consisting of dimers of polypeptide monomers selected from the group consisting of P3 OF 31-34 subunit A, subunit B, subunit C, subunit D and subunit E. The process comprises the steps of culturing in suitable culture media one or more cell lines transformed with nucleic acid sequences encoding one or more polypeptides selected from the group consisting of P3 OF 31-34 subunit A, subunit B, subunit C, subunit D and subunit E. Dimers are then formed of the polypeptide monomers by linking them with at least one disulfide bond and the dimers so formed are then isolated.

Proteins or polypeptides that are or can be converted to osteogenically active species which are

immunologically related to the P3 OF 31-34 proteins or subunits or fragments thereof are also considered to be within the scope of the present invention. Active entities, referred to herein as "active polypeptides", include any portion of the proteins or polypeptides which are the subject of the present invention and their functional derivatives which can be produced by conventional procedures such as chemical synthesis or recombinant DNA techniques. Active polypeptides further include deletions from, or insertions or substitutions of residues within the amino acid sequence of the osteogenic proteins and subunits. Combinations of deletion, insertion and substitution may also be made to arrive at the final construct, provided that the final construct possesses osteogenic activity. Derivatives of such active polypeptides can include, for example, chemically or enzymatically modified polypeptides; fusion proteins; or polypeptides bound to a suitable carrier substance such as a polymer.

Natural sequence polypeptide subunits present in P3 OF 31-34 of one or more mammalian species or analogs and variants thereof may be prepared by direct chemical synthesis of polypeptide or by expression of DNA prepared by site-directed mutagenesis of subunit DNA or by chemical synthesis of oligonucleotide and assembly of the oligonucleotide by any of a number of techniques prior to expression in a host cell. [See, e.g., Caruthers, U.S. Patent No. 4,500,707; Balland, et al., Biochimie, 67, 725-736 (1985); Edge, et al., Nature, 292, 756-762 (1981)]. Messenger RNA encoding P3 OF 31-34 or analogs thereof may also be expressed in vitro. Changes in activity levels are measured by the appropriate assay. Modifications of such protein properties as redox or thermal stability, hydrophobicity, susceptibility to proteolytic degradation, or the tendency to aggregate with carriers

or into multimers are assayed by methods well known to those of ordinary skill in the art.

Prokaryotic microorganisms (such as bacteria) and eukaryotic microorganisms (such as yeast) may be employed as host cells according to the present invention. S. cerevisiae, or common baker's yeast, is the most commonly used among eukaryotic microorganisms, although a number of other strains are commonly available. For expression in bacteria and yeast, cloning and expression vectors are well known to those skilled in the art, such as lambda phage and pBR322 in E. coli and YRp7 in S. cerevisiae.

Cells derived from multicellular eukaryotes may also be used as hosts. Cells from vertebrate or invertebrate eukaryotes may be used, and those skilled in the art know of appropriate expression vectors for use therein, such as SV40 retroviral and papilloma viral vectors for mammalian host cells, NPV vectors for invertebrate host cells and Ti vectors for plant cells.

The present invention further discloses methods of using one or more of the proteins and/or active polypeptides and/or immunologically related entities as pharmaceutical agents for the stimulation of bone growth in mammals. Pharmaceutically acceptable compositions comprised of one or more of the proteins and/or active polypeptides and/or immunologically related entities in combination with a pharmaceutically acceptable carrier are also disclosed herein. Such compositions can optionally contain other bioactive materials or other ingredients which aid in the administration of the composition or add to the effectiveness of the composition.

As used herein, the term "immunologically related" is meant to include any polypeptide which shows binding and/or recognition to antigen-binding sites in antibodies raised or manufactured against the protein.

The term "osteogenesis" means formation of new bone or induction of growth of pre-existing bones at specific sites in response to local administration (for example, implantation of an active preparation in a pharmaceutically acceptable manner). The term "osteogenic amount" refers to an amount of the osteogenic protein and/or active polypeptide and/or immunologically related entity sufficient to provide the desired effect. The term "osteogenically active" or "osteogenic" means that the preparation has the capability to promote or induce osteogenesis.

In addition, two unrelated protein preparations designated herein as P2 and P4 have also been isolated from bone of several different mammalian species. A family of P2 proteins, each member isolated from a particular mammalian bone source, has been characterized. A typical P2 protein isolated from calf bone has an apparent molecular weight of 30,000 to 33,000 daltons, but is incapable of inducing osteogenesis in the absence of the osteogenic protein associated with the P3 protein preparation. Immunologically related P2 protein has also been isolated according to the procedure essentially as described herein from human bone.

In a similar manner, a family of P4 proteins has been isolated according to the procedures described herein. In the stage of purification accomplished from calf bone, the P4 preparation consists of two major components which are incapable of inducing osteogenesis in the absence of the osteogenic protein associated with the P3 protein preparation, both having an apparent molecular weight of about 16,000 to 18,000 daltons and are characterized by amino terminus amino acid sequences as described later herein. Immunologically related members of this P4 protein family which are also incapable of inducing osteogenesis in the absence of the

osteogenic protein have been isolated from human bone according to the procedures described herein.

The application of the osteogenic factors can be conveniently accomplished by administering, such as by implanting, a lyophilized preparation or suspension of one or more of the osteogenic proteins and/or one or more active polypeptide and/or one or more immunologically related entities in sufficient quantity to promote osteogenesis at the desired site. Alternatively, pharmaceutically acceptable compositions can be used which are comprised of one or more of the osteogenic proteins and/or one or more of the active polypeptides and/or one or more of the immunologically related entities described herein and a pharmaceutically acceptable matrix such as collagenous proteins or matrix material derived from powdered bone extracted with strong denaturing agents, or other pharmaceutically acceptable carriers.

The following examples are included to further illustrate the invention but are not to be construed as limitations thereon.

Example 1

Isolation Of The Osteogenic Factors

Bone Processing

In a typical preparation, long bones (ends of long bones) from a mammal (for example, ankles from calves, femur heads of vertebral column from human bones, the total tibia and fibula from rats) are processed and demineralized using well known conventional procedures such as those described in Urist, M.R., U.S. Patent No. 4,294,753 (1981). These and all other references cited herein are incorporated herein by reference.

A convenient method of processing and demineralizing bone is as follows:

The periosteal layer surrounding the bone (preferably the bone is obtained from a young mammal and kept refrigerated until processing) is removed by mechanical means and then the marrow from the central cavity of the bone is removed by washing with cold water. The bone is pulverized into small particles [generally 1 to 2 millimeters (mm) in diameter] by conventional means, for example, using a Wiley mill. The particles are then washed extensively with a buffered saline solution such as a 0.15 M NaCl-0.1 M Tris·HCl buffer (pH 7.0) to remove most of the lipids and remaining blood. The particles are further reduced in size by shearing, for example, using a polytron homogenizer (Brinkman Instruments) so that particles of approximately 500 microns (μ) in diameter or less are obtained. The homogenized particles are washed with buffered saline such as that noted above and water, then with ethanol and finally with ether. The washed homogenized particles are then vacuum or air dried; this "bone powder" can be stored at -80°C for prolonged periods of time.

For efficient demineralization and protein extraction, the bone powder is sieved to obtain particles having a size range of about 75 to 500 μ in diameter. Demineralization (that is, the removal of calcium phosphate from the bone matrix) is achieved by repeated washes with a hydrochloric acid (HCl) solution, for example, by stirring bone powder for one hour with about 10 to 15 milliliters (ml) of 0.5 normal (N) HCl per gram (g) dry weight of bone powder, decanting the liquid and then repeated this process three or four times. The demineralized bone powder is then washed extensively with deionized distilled water until the pH approaches neutrality. The water is removed from the demineralized bone powder by washing with ethanol, then ether, and then drying. The demineralized bone powder

can be stored at ultralow temperatures (for example, -20° to -80°C). Demineralization of the bone powder can also be accomplished using other well known procedures, for example, using a chelator such as ethylenediaminetetraacetic acid.

To determine if the treated bone powder is sufficiently demineralized after HCl treatment to be ready for the extraction of the bone-matrix proteins, the water-rinsed powder is tested for mineral content [(that is, calcium content), for example, by the method of von Kossa, see J. von Kossa, Ziegler's Beitr. 29, 163 (1901)]. When the von Kossa stain is negative, the treated bone powder is sufficiently demineralized to be ready for the extraction of proteins.

Extraction and Separation of Proteins From Demineralized Bone Powder

Demineralized bone powder, prepared as described above, is extracted by constant stirring with an aqueous solution of about 2 to 8 molar (M) guanidium-hydrochloride (GuHCl) in a buffer such as Trizma-hydrochloride (Tris·HCl) at or near pH 7.0 for a time sufficient to extract the desired proteins. Preferably, the extraction is performed by stirring the demineralized bone powder with 4 M GuHCl-0.01 M Tris·HCl buffer (pH 7.0) in the presence of a proteolytic enzyme inhibitor such as phenylmethylsulfonyl-fluoride for 8 to 12 hours (hrs) between about 4° to 20°C. The proteins from demineralized bone powder can be extracted by contacting the demineralized bone powder with an appropriate GuHCl-Tris·HCl buffer for a time sufficient to obtain substantial quantities of the desired proteins. In a typical extraction of 100 grams of demineralized calf bone powder, approximately 1500 milligrams (mg) of total proteins are extracted in a three day extraction period with 4M GuHCl-0.01 M

Tris·HCl buffer (pH 7.0). In the process of the present invention, it has been found that more than 80 percent (%) of the total proteins obtained in a three day extraction can be extracted in the first 8 to 12 hrs with a 4 M GuHCl-0.01 M Tris·HCl buffer (pH 7.0). During the first 8 to 12 hrs of extraction, typically more than 95% of the total low molecular weight protein population that can be obtained in a three day extraction is recovered. Most osteogenic activity is associated with these low molecular weight proteins. About 15 ml of the 4 M GuHCl-0.01 M Tris·HCl buffer (pH 7.0) solution is used per gram dry weight of demineralized bone powder. After the extraction period is complete, the extract is filtered, for example, over Whatman paper, and the filtrate concentrated by conventional procedures; in typical experiments, an Amicon ultrafiltration apparatus (Amicon Corporation, Danvers, Massachusetts) with a membrane filter with molecular cut-off size of approximately 5,000 daltons is used for the concentration step (that is, the membrane retains molecules having a molecular weight greater than approximately 5,000 daltons, for example, an appropriate Diaflo® ultrafiltration membrane such as YM-5).

The various buffers, for example, the 4 M GuHCl-0.01 M Tris·HCl buffer, the solutions, for example, the 0.5 N HCl solution, described herein are aqueous buffers or solutions in which the indicated materials are present in water at the indicated concentration. The protein components of the concentrated protein solution were fractionated using various conventional chromatographic techniques including high performance liquid chromatography (HPLC) as follows:

The initial protein fractionation was conveniently accomplished by chromatography on a Sepharose CL-6B (Pharmacia Chemicals, New Jersey)

column. In a typical experiment, the proteins extracted as described herein are concentrated by ultrafiltration to a concentration of about 25 to 40 mg/ml. The concentration of proteins in various extract preparations and column fractions were usually estimated by conventional means such as spectrophotometric measurement of the absorbence of the solutions at 280 nanometers (nm). An appropriate amount of protein concentrate (an amount providing approximately 500 mg of protein) was applied to a 5 centimeter (cm) x 90 cm Sepharose CL-6B column equilibrated with 4 M GuHCl-0.01 M Tris·HCl buffer (pH 7.0). The column is eluted with the 4 M GuHCl-0.01 M Tris·HCl buffer (pH 7.0) at a hydrostatic pressure head of between about 50 to 100 cm and individual fractions of 15 to 20 ml volume collected. A typical elution profile under the above conditions was obtained by measuring the absorbence of individual fractions at 280 nm and is shown in Figure 1.

The bone inducing activity of various fractions eluted from the Sepharose CL-6B column was measured, using the bone induction assay system described herein, and indicated that the pool of fractions identified as "C" in Figure 1 contained the factors responsible for the osteogenic activity. Pool C, which consisted of pooled fractions V, VI and VII, was concentrated using conventional procedures. In a standard extraction, pool C obtained from the elution of the total proteins on the Sepharose CL-6B column represents about 40% of the total proteins obtained in an 8 to 12 hr extraction of demineralized calf bone powder with 4 M GuHCl-0.01 M Tris·HCl buffer (pH 7.0). Further fractionation was then achieved by chromatography on a Sephacryl S-200 (Pharmacia Chemicals, New Jersey) column. In a typical experiment, 75 to 100 mg of proteins from pool C are applied at a

concentration of approximately 25 mg/ml to a 2.2 cm x 115 cm Sephacryl S-200 column and the column eluted with 4 M GuHCl-0.01 M Tris·HCl buffer (pH 7.0) under a hydrostatic pressure head of between about 50 to 75 cm and individual fractions of approximately 4 ml in volume collected. A typical elution profile which was obtained under the above conditions is shown in Figure 2.

Fractions from the Sephacryl S-200 column were pooled (see Figure 2) and the resulting pooled materials arbitrarily identified as alpha (α), beta (β), gamma I (γI), gamma II (γII) and delta (δ).

Analysis of the proteins, using conventional discontinuous polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate visualizing the protein bands by staining with coomassie blue [Laemmli, U.K., Nature, Vol. 227, pp. 680-685 (1970)], contained in the respective alpha through delta pools allowed identification of several proteins. It was found that the alpha pool contained minor protein components of molecular weight higher than 50,000 daltons; the beta pool contained a major species at 38,000 to 40,000 daltons, some minor higher molecular weight contaminants, and small quantities of lower molecular weight protein species migrating between 14,000 and 30,000 daltons; the gamma I and gamma II pools contained four major size class species migrating at 31,000 to 35,000 daltons, at 22,000 to 25,000 daltons, at 16,000 to 18,000 daltons, and at 12,000 to 14,000 daltons; the delta pool contained mostly proteins in the 12,000 to 14,000 dalton range.

Measurement of activity in the bone induction assay essentially as described herein indicated that the gamma I and gamma II pools contained factors inducing bone formation.

To simplify the discussions concerning the final purification of the osteogenic factors, a list of the protein species found in the beta, gamma and delta pools is presented in Table 1. As indicated previously, each of the respective major protein species was assigned an identifying code (P1, P2 and the like) as indicated in Table 1.

Table 1

Major Species		Minor Species	
Assigned Name	Estimated Molecular Weight X 10^{-3}	Assigned Name	Estimated Molecular Weight X 10^{-3}
P1	38-40		
P2	30-33		
		PA	28-30
		PB	24
P3	22-24		
		PC	19
P4	16-18		
P5a	13-14		
P5b	14*		
		PD	12

All primary molecular weight assignments of protein species are based on mobilities in discontinuous polyacrylamide gel electrophoresis with 13% acrylamide at pH 8.8 in the resolving gel in the presence of sodium dodecyl sulfate and a reducing agent. The minor protein species represented less than 10 to 15 percent of the total material in the respective samples analyzed on gels. *P5b migrates at about 10,000 daltons under non-reducing conditions which serves to distinguish P5a from P5b.

Reverse Phase HPLC Purification of the Osteogenic Preparation

A further purification step was carried out by reverse phase HPLC of the partially purified protein preparations, obtained from Sephacryl S-200 column chromatography, using a Beckman Altex HPLC controlled by

a Model 421 microprocessor unit. Two approaches have been used.

A characteristic feature of some of the isolated proteins, especially the P3 protein family described herein and the osteogenically active protein preparation copurifying therewith is the lack of solubility in the absence of a strong dissociating agent such as GuHCl. In addition, when multiple protein species were simultaneously present in a pool, the removal of GuHCl resulted in a coprecipitation of other proteins along with the P3 proteins including the P3 OF 31-34. A method was, therefore, developed where narrow pools consisting of only one or two major proteins were obtained from the Sephacryl S-200 column and used as the starting material for further purification by HPLC. In addition, in order to maximize the retention of proteins in solution, pools such as the ones described above were dialyzed directly against an aqueous solvent containing 0.1% trifluoroacetic acid (TFA) supplemented with acetonitrile (ACN) at concentrations of between 10% to 15% by volume. A conventional dialysis membrane tubing with molecular weight cut-off size of 3,500 daltons or lower is conveniently used in this procedure. Proteins soluble in the TFA:ACN solvent could then be conveniently obtained by removal of the insoluble material from each dialyzed pool by centrifugation. The soluble proteins at this point could be chromatographed on a reverse phase HPLC column such as the Protesil 300 octyl column described herein. In a typical experiment, the TFA:ACN soluble proteins obtained from the peak fractions in this manner were applied to a 0.46 cm x 25.0 cm Protesil 300 octyl column (Whatman) of 10 micron particle size equilibrated with 0.1% TFA:10% ACN. Proteins bound to the column under these conditions were eluted at a flow rate of 60 ml/hr using a linear 10% to 80% ACN gradient developed over

45 minutes. In a typical experiment, as indicated in Figure 3A, P2 and P1 proteins were sequentially recovered with increasing ACN concentrations (depicted by the dashed line) from the gamma I peak. Similarly, P1 protein can be obtained from the beta peak while P5a and P5b are obtained from the delta peak. The P3 protein and the osteogenically active protein associated therewith elute between the gamma I and gamma II regions of the Sephacryl S-200 column. The P3 and P3 OF 31-34 protein preparation is found in both the soluble and the insoluble materials obtained by dialysis of appropriate functions against TFA:ACN. The lack of solubility of the P3 and P3 OF 31-34 proteins thus yields osteogenically active protein in the presence of substantially purified P3 protein in the insoluble material. The P3 and P3 OF 31-34 proteins retained in solution in the TFA:ACN solvent can be further purified by reverse phase HPLC essentially as described above.

The second procedure to purify proteins to an essentially homogeneous state was designed to take advantage of the high degree of insolubility of certain proteins in the 35,000 to 14,000 dalton range, especially when they are present together at high concentrations (for example, approximately 10 mg/ml). In this procedure, proteins eluting in the gamma I and gamma II pools from the Sephacryl S-200 column chromatography (that is, the pools where the bone inducing activity is found) were concentrated to approximately 10 mg/ml. The material was rapidly dialyzed [for example, six changes each of 4 liters every 2 to 3 hrs, (using dialysis tubing with a molecular cut-off size of 2,000 daltons)] against deionized distilled water at 15° to 23°C. Precipitated proteins were collected by centrifugation and washed several times with deionized distilled water keeping the concentration of protein at higher than 10 mg/ml of

washing water. The principal constituents of this precipitated material were found to be P2, P3, P4 and P5a; small amounts of P1 protein was found in variable quantities in some cases. The final pellet was dissolved in 0.1% TFA with 15% ACN and the solubilized material was applied to a Protesil 300 octyl column. Increasing ACN concentration eluted the P2, P4, P5a and P3 proteins as shown in Figure 3B, a typical elution profile.

Each of the major protein species described in Table 1 was further purified by rechromatographing on the Protesil 300 octyl column. Pools of fractions obtained as indicated in Figure 3 were concentrated by lyophilization and redissolved in 0.1% TFA and about 10 to 20% ACN depending upon the particular lyophilized material and reapplied to the Protesil 300 octyl column. The proteins were eluted from the column using a linear 10% to 80% ACN gradient at a flow rate of 60 ml/hr under conditions as previously described herein except that the proteins were eluted over a longer period, thus resulting in numerous individual fractions. The purity of each of the protein fractions was determined using conventional discontinuous PAGE. Those fractions which showed only one major species were used for further chemical and biological characterizations. Typically, these fractions were lyophilized and stored as lyophilized powders.

Figure 4 depicts the results of a typical discontinuous gel electrophoretic analysis on sodium dodecyl sulfate-polyacrylamide gels. The analysis was performed on a discontinuous polyacrylamide gel system in the presence of sodium dodecyl sulfate and a reducing agent where the resolving gel was 13% in acrylamide and 0.35% in bis-acrylamide crosslinker at a pH of 8.8. The gel was run at 50 volts for 30 minutes followed by 7 hrs at 100 volts. Protein bands were visualized by staining

with coomassie brilliant blue R. Columns 1 and 8 depict gels with the following standard molecular weight markers: 95,000 (phosphorylase A), 68,000 (bovine serum albumin), 43,000 (ovalbumin), 31,000 (carbonic anhydrase), 21,000 (soybean trypsin inhibitor), and 14,000 (ribonuclease); columns 2, 3, 4, 5 and 6 show, respectively, the P1, P2, P3 (including the P3 OF 31-34 protein), P4 and P5 protein (CP1 through CP5) from demineralized calf bone powder; and column 7 the P3/osteogenic protein (HP3) from demineralized human bone powder. Portions shaded with oblique lines are bands of low concentration.

The calf and the human protein preparations comprising the P3/osteogenic proteins each, when implanted in rats following the bioassay system described herein, induces the formation of bone at the implant site in approximately 3 weeks. It appears that the osteogenic proteins which copurify with the members of the P3 protein family isolated from different mammals will show osteogenic activity in mammals in general. Thus, the P3 proteins represent a family of immunologically related proteins which copurify with the primary osteogenic factors according to the above methods.

Bone Induction Assay System

To determine the osteogenic activity of test protein fractions or proteins a procedure such as the following can be used. Bone matrix powder (75 to 500 μm size) is demineralized as described herein and then extracted sequentially three times, each with 15 to 20 ml of 4 M GuHCl per gram of demineralized bone powder. The extracted matrix is extensively washed with water, followed by ethanol and ether and then the powder is dried. This powder, when implanted in a test animal, such as a rat, does not induce osteogenesis and is

called inactive bone matrix (IBM). In order to measure the activity of a protein preparation, the IBM powder is mixed with an aqueous solution or suspension of the protein and the water removed by lyophilization. The reconstituted matrix is then packed in gelatin capsules and implanted subcutaneously near the thigh muscles of young (one to two months old) rats. Varying amounts of protein preparations are used together with a constant amount of IBM in each capsule to determine the efficacy of the different protein preparations. Osteogenic activity in each implant is estimated by two approaches, (a) measuring the level of the enzyme alkaline phosphatase in the implant tissues at 17 to 20 days following implantation and (b) performing a histologic examination of a 5 to 7 micron thick section of the tissue developed at the implant site following staining of paraffin-fixed sections of this tissue with toluidine blue (stains cartilage matrix and bone matrix), hematoxylin-eosin (resolves fibrous, cartilaginous and bone tissues) and von Kossa silver stain (for calcified matrix of bone tissue).

The level of alkaline phosphatase is measured since active bone formation is characteristically preceded by a significant surge of this enzyme and continued formation of bone is accompanied by a stable elevated level of alkaline phosphatase activity compared to that found in non-bone fibrous tissue surrounding the implants. An approximate quantitation of the levels of bone inducing activity in a protein preparation has been obtained by quantitating the level of alkaline phosphatase per unit weight of implant tissue. In practice, the implant tissue is homogenized in an appropriate buffer such as Tris-saline, dissociated with a nonionic detergent and the solubilized enzymes that are released from the tissue are obtained by removing the debris using centrifugation. The levels of alkaline

phosphatase are quantitated by measuring the conversion of paranitrophenylphosphate to paranitrophenol catalyzed by dilutions of the test extract and calculating from a standard curve of known enzyme activity.

In bioassay studies, protein pools from the Sephacryl S-200 column were reconstituted with IBM and implanted subcutaneously in rat thighs. Measurement of alkaline phosphatase activity and histological evaluation of sections of explants removed 17 to 20 days after implantation, showed that the P1 and the P5a-P5b proteins do not have bone inducing activity. The bioassay studies indicated the presence of maximum osteogenic activity in proteins in pools gamma I and gamma II. The three major components of the gamma fractions, that is, the P2 protein, the P3 and P3 OF 31-34 protein and the P4 proteins were purified using reverse phase HPLC as described above. The purified proteins, either singly or in a complete mixture, were reconstituted with inactive bone matrix and a bone induction assay performed. The results are shown in Table 2.

Table 2

	Alkaline Phosphatase (units/g)	Histology
IBM* Alone	<5	Fibrous Tissue
IBM + 750 µg P2 protein	<5	Fibrous Tissue
IBM + 750 µg P3 protein (including the P3 OF 31-34 protein)	78	New Bone
IBM + 1000 µg P4 protein	<5	Fibrous Tissue (a small trace of cartilage)
IBM + 250 µg each of P2, P3 (including the P3 OF 31-34 protein) and P4 proteins	63	New Bone

*"IBM" means Inactive Bone Matrix.
"<" means less than.

The data in Table 2 indicate that the P3 protein (including the P3 OF 31-34 protein) alone induced the formation of bone. Implants containing the P3 and P3 OF 31-34 preparation developed into tissues that contained high levels of alkaline phosphatase enzyme activity. In contrast, implants prepared by reconstituting with either the P2 or the P4 preparation failed to produce detectable bone. When all three preparations were used in combination, significant bone formation was observed and high levels of alkaline phosphatase enzyme were obtained with one-third the amount of P3 protein preparation (as compared to the P3 protein implant alone). It thus appears that at low concentrations of P3 protein preparation including P3 OF 31-34, the presence of the P2 and/or the P4 protein provides enhancement of osteogenesis induced by the P3 protein preparation.

In using the active preparations described herein, an osteogenic amount of the protein and/or active polypeptide and/or immunologically related entity, with or without a pharmaceutically acceptable carrier, is administered at or in the proximity of the site in the mammal at which bone induction is desired. Administration will depend on the age, condition, sex and other characteristics of the subject to be treated. Preferred administration is by implantation, local injection or time controlled delivery using microcapsules, or other devices. Dosages will depend on the site and configuration of the area to be healed, such as, for example, a fracture zone. For example, a 5 cubic millimeter bone chip can be obtained with about 100 to 200 micrograms (µg) of P3 protein administered or implanted locally in the form of an implant in about 100 mg of IBM.

Active preparations can include other suitable bioactive materials such as growth factors, chemotactic agents, steroids, antibiotics, anti-inflammatory agents and the like.

Also provided by the present invention are alternative methods whereby the osteogenic protein present in the preparation purified according to Example 1 may be treated so as to isolate the P3 OF 31-34 protein which is of extremely high purity and osteogenic potency.

The process of Example 1 for obtaining the P3 family of immunologically related protein included a demineralization step, a guanidine extraction step, a size fractionation step in non-reducing denaturing solvents, a dialysis step and a reverse phase HPLC step. An improvement of the size fractionation step involved the use of molecular sizing filters which could fractionate very large volumes of material and yield a molecular weight cut between 5,000 or 10,000 daltons and

100,000 daltons (5K-100K or 10K-100K). A second improvement of the size fraction step relied on the pooling of protein fractions eluting within narrower molecular weight ranges from a gel filtration chromatography column (Sephacryl S-200) run in a non-reducing denaturing solvent. Using the S-200 column, the osteogenic activity was eluted within the region corresponding to a molecular weight range of 25,000-38,000 daltons, whereas the γI and γII pools used to purify the P3 protein had a molecular weight range of 14,000-40,000 daltons wherein the material immunoreactive with the antibodies directed against the major immunogenic determinants in the P3 protein migrated between the molecular weight range of 14,000-25,000 daltons (Figure 8).

With use of these improvements to the process, the osteogenic activity eluted from reverse phase HPLC columns within the same concentrations of acetonitrile as those concentrations of acetonitrile required to elute the P3 family of related proteins. Reverse phase HPLC of the S-200 active pool (eluting within the molecular weight range of 25,000-38,000 daltons) allow further resolution of these components of P3 protein which could be subfractionated using additional or different fractionation steps.

Fractionation of the osteogenically active pool of 25,000-38,000 dalton proteins using DEAE ion-exchange chromatography columns (Pharmacia Chemicals, New Jersey) showed that the osteogenic activity does not bind to the DEAE column at pH 6.5 and thus could be separated from material which bind to DEAE-column. Further fractionation of the osteogenically active protein preparation has been achieved using chromatofocusing columns (Pharmacia Chemicals, New Jersey) whereby the activity is recovered at an apparent pH of 7.5 or greater. This extended purification work

of osteogenically active molecules in P3 proteins has also indicated that the osteogenic activity was distinct from the TGF-beta and TGF-beta immunoreactive material. The osteogenic activity bound binds to a Mono-S FPLC (Fast Protein Liquid Chromatography) column (Pharmacia Chemicals, New Jersey) equilibrated at pH 6.5 and can be eluted at a NaCl concentration greater than that required to elute the TGF-beta or TGF-beta immunoreactive material. It was also found that the osteogenic activity in the S active pool eluted from Mono-S column again could be characterized by its elution from a reverse phase HPLC column within the same concentrations of acetonitrile required to elute the P3 proteins.

Example 2

Alternative Method for Purification of Bovine Osteogenic Factors

This example illustrates an alternative method providing the complete purification of the osteogenically active 31,000-34,000 dalton components of the P3 protein from larger quantities of bone powder and demonstrates that these protein components which are minor constituents of the P3 protein are osteogenically active in the essential absence of the major 22,000-24,000 dalton protein component. According to this example, bovine osteogenic factors were isolated from demineralized calf bone powder according to the procedure disclosed in Figure 9. Approximately 200 pounds of diaphysial sections of calf bone were scraped clean of connective tissue and marrow was removed. The demarrowed sections were ground to a powder and washed with approximately 2100 liters of cold deionized water. The bone powder was allowed to settle during the water washes and the suspended connective tissue fragments were removed with the supernatant and discarded.

The bone powder was suspended in a total of approximately 570 liters of cold 0.5 M HCl for about 2 hours and was then allowed to settle. The HCl was removed with the supernatant and discarded. The remaining HCl was removed by washing the bone powder with approximately 700 liters of cold deionized water, followed by approximately 350 liters of cold 0.1 M Tris, pH 7, solution. The demineralized bone powder (demineralized bone) was allowed to settle and the supernatant was discarded.

The demineralized bone powder was suspended in approximately 140 liters of cold 4 M guanidine hydrochloride containing 0.01 M Tris, pH 7, and 0.001 M EDTA for about 20 hours. The extracted bone powder was removed by filtration and discarded. The supernatant (guanidine extract) was saved.

The guanidine extract was filtered through Amicon spiral cartridges with an average molecular weight cutoff of 100,000 daltons. The 100,000 dalton filtrate (100K filtrate) was then concentrated through Amicon spiral cartridges with molecular weight cutoffs of 10,000 daltons. The 10,000 dalton retentate (10K retentate) was saved and assayed for pH, conductivity, total protein content by BCA colorimetric protein assay (Pierce Chemicals, Rockford, Illinois), resolution of protein constituents in the preparations using reducing SDS-PAGE followed by silver staining or coomassie blue staining and determination of the osteogenic activity using the rat implant assay disclosed below in Example 3.

The 10K retentate was exchanged into 6 M urea containing 50 mM 2-(N-morpholino)ethanesulfonic acid (MES), pH 6.5, by diafiltration with an Amicon spiral cartridge with a molecular weight cutoff of 10,000 daltons.

The diafiltered extract was adjusted to a pH of 6.5 using 5 M NaOH and a conductivity of 10 mS/cm using 5 M NaCl and applied to a 0.4 liter S-Sepharose column (Pharmacia Chemicals, New Jersey) equilibrated with 6 M urea containing 50 mM MES, pH 6.5, adjusted to conductivity of 10 mS/cm. The column was washed with 2.4 liters of 6.0 M urea containing 50 mM MES, pH 6.5, adjusted to a conductivity of 10 mS/cm to elute the unbound proteins. The S-Sepharose active pool (SS Pool) was eluted with 1.2 liters of 6.0 M urea containing 50 mM MES, pH 6.5, and 0.5 M NaCl. The S-Sepharose active pool was concentrated using membrane filters with an average molecular weight cutoff of 10,000 daltons. The pH and conductivity of the preparation were determined, the total protein content was measured by BCA protein assay, the protein constituents were analyzed using SDS-PAGE followed by silver staining and the osteogenic activity was determined using the rat implant assay.

The S-Sepharose active pool was gel filtered with a 3 liter Sephadex G-25 column (Pharmacia Chemicals, New Jersey) equilibrated with 6 M urea containing 20 mM ethanolamine, pH 9.5. The first protein peak containing the active pool (G-25 Pool) was eluted with 3 liters of 6 M urea containing 20 mM ethanolamine, pH 9.5.

The G-25 Pool was applied to a 0.7 liter Q-Sepharose column (Pharmacia Chemicals, New Jersey) equilibrated with 6 M urea containing 20 mM ethanolamine, pH 9.5. The column was washed with 2.1 liters of 6 M urea containing 20 mM ethanolamine, pH 9.5, to elute the unbound proteins. The osteogenically active protein pool (QS Pool) was eluted from Q-Sepharose column with 1.4 liters of 6 M urea containing 20 mM ethanolamine, pH 9.5, and 0.2 M NaCl. The QS Pool was adjusted to a pH of 6-7 with glacial

acetic acid and concentrated using membrane filters with an approximate molecular weight cutoff of 10,000 daltons. The QS Pool was assayed for pH and conductivity; the total protein content was determined by BCA protein assay, the protein constituents were analyzed by reducing SDS-PAGE followed by silver staining and the osteogenic activity was measured using the rat implant assay.

The QS Pool was then applied to a preparative C-18 HPLC column equilibrated with a buffer containing, by volume, 70% Buffer A (Buffer A is 0.05% trifluoroacetic acid in water) and 30% Buffer B (Buffer B is 0.025% trifluoroacetic acid in acetonitrile). Bound proteins were eluted using a linear gradient of 30% to 60% acetonitrile in 120 minutes. As previously characterized for P3 protein of example 1, the osteogenic activity (Prep HPLC Pool) eluted within the concentrations of 35% to 45% acetonitrile. The Prep HPLC Pool was lyophilized and resuspended in 1 ml of water. The Prep HPLC Pool was assayed for pH and conductivity; the total protein content was determined by BCA protein assay, the protein constituents were analyzed by reducing SDS-PAGE followed by silver staining and the osteogenic activity was measured using the rat implant assay.

The Prep HPLC Pool was adjusted to a protein concentration of 0.5 mg/ml in 6 M urea containing 50 mM Tris, pH 7.5-8.0, 20 mM ethanolamine and 0.5 M NaCl and was applied to a 5-10 ml Chelating Sepharose 6B column (Pharmacia Chemicals, New Jersey) charged with Cu 2+ and equilibrated with 6 M urea containing 50 mM Tris, pH 7.5-8.0, 20 mM ethanolamine and 0.5 M NaCl. The column was washed with 5 column volumes of equilibration buffer followed by 10 column volumes of 6 M urea containing 50 mM Tris, pH 7.4-7.8, to elute the unbound proteins. Bound proteins were eluted with 10 column

volumes of 6 M urea containing 50 mM Tris, pH 7.4-7.8, and 4 mM imidazole. The osteogenic activity (CC Pool) was eluted from the copper chelate column with 10 column volumes of 6 M urea containing 50 mM Tris, pH 7.4-7.8, and 15 mM imidazole. The CC Pool was assayed for total protein as estimated by absorbence at 280 nm, and its osteogenic activity was measured using the rat implant assay.

The CC Pool was adjusted to 25% ammonium sulfate and loaded onto a 1-3 ml column of Phenyl-Sepharose (Pharmacia Chemicals, New Jersey) equilibrated with 6 M urea containing 25% ammonium sulfate, 50 mM Tris pH 7.4-7.8. The column was washed with 10 column volumes of 6 M urea containing 25% ammonium sulfate, and 50 mM Tris pH 7.4-7.8, to elute the unbound proteins. Bound proteins were eluted with 10 column volumes of 6 M urea containing 15% ammonium sulfate, 50 mM Tris pH 7.4-7.8. The osteogenic activity (PS Pool) was eluted from the Phenyl-Sepharose column with 6 M urea containing 50 mM Tris pH 7.4-7.8, was assayed for total protein as estimated by absorbence at 280 nm, and its osteogenic activity was measured using the rat implant assay.

The PS Pool was applied to a semi-preparative or analytical C-18 HPLC column equilibrated with a buffer containing, by volume, 70% Buffer A and 30% Buffer B, as described previously (Buffer A is 0.05% trifluoroacetic acid in water and Buffer B is 0.025% trifluoroacetic acid in acetonitrile). Bound proteins were eluted using a linear gradient of 30% to 60% acetonitrile. As was previously characterized, the osteogenic activity (HPLC Pool) eluted within the concentrations of 35% to 45% acetonitrile. The HPLC Pool was assayed for total protein as estimated by absorbence at 229 nm and its osteogenic activity was measured using the rat implant assay.

Characterization of Bovine Osteogenic Factors

In the following examples, preparations of bovine osteogenic factors were characterized according to various procedures.

Example 3

Biological Activity

The induction of bone matrix was measured using a rat implant assay as generally described by Sen, Walker and Einarson, 1986. In Development and Diseases of Cartilage and Bone Matrix, eds. A. Sen and T. Thornhill, pp. 201-220. Alan R. Liss, New York and Sampath and Reddi, Proc. Natl. Acad. Sci. U.S.A., 80:6591-6595 (1983). Approximately 70-100 mg of inactive bone matrix (bone collagen) was mixed with an aqueous solution of osteogenic protein preparation and the water removed by lyophilization. The dried coated granules were packed in gelatin capsules (Eli Lilly #5) and each capsule was subcutaneously implanted near the thigh muscles in each back leg of male Long Evans rats. The implanted rats were sacrificed 21 to 28 days following implantation and the implant tissue was surgically removed and placed in Bouin's Solution. The specimens were then decalcified and processed for toluidine blue stained sections. Histomorphology and percent ossification was determined by examination of the stained sections. Potency is defined by the amount of protein (mg) required for implantation with inactive bone matrix yielding at least 10% of the area of the stained sections occupied by osteoid activity.

Table 3

Purification of Osteogenic Factors

Sample	Total Protein	Potency in Rat (mg/implant)
Guanidine Extract	130,000-170,000 mg	
10K Retentate	6,000-15,000 mg	10.0
S-S Pool	300-900 mg	1.0
QS Pool	70-250 mg	0.25
Prep HPLC Active Pool	4-12 mg	0.05
CC Pool	2-5 mg	0.025
PS Pool	0.5-1 mg	0.01
HPLC Active Pool	0.01-0.05 mg	0.001

The increase in potency of the various osteogenically active protein preparations obtained using purification steps according to Example 2 is shown in Table 3, above, with the HPLC Active Pool having a potency of 0.001 mg/implant which is significantly higher than the P3 protein produced according to Example 1.

Example 4

Estimation of Molecular Weight of Osteogenic Activity

Osteogenically active protein preparations, obtained using various purification steps described in Example 2, were suspended in SDS sample dilution buffer (in the absence of reducing reagents) and applied to a 10% SDS polyacrylamide gel and electrophoresed. Molecular weights were determined relative to either prestained molecular weight standards (Bethesda Research Labs, Gaithersburg, Maryland) or non-prestained molecular weight standards (Bio-Rad, Richmond,

California). After completion, the gel lanes were sliced into pieces. Each piece was electroeluted to extract the protein. The eluted protein was precipitated with acetone, resuspended in guanidine hydrochloride, dialyzed against water, lyophilized onto inactive bone matrix and implanted into rats to assay osteogenic activity according to Example 3. In this gel system, the osteogenic activity was eluted from gel slices corresponding to the apparent molecular weight range of 28,000-34,000 daltons.

Example 5

Molecular Weight of Purified Osteogenic Factors

Purified osteogenically active protein preparation as obtained in the HPLC Active Pool of Example 2 were suspended in SDS dilution buffer in the absence of reducing reagents (-DTT), electrophoresed on 12.5% or 15% SDS polyacrylamide gels and the protein bands visualized by silver staining. Molecular weights are determined relative to non-prestained molecular weight standards (Bio-Rad). This gel system revealed that the HPLC Active Pool contained protein bands which migrate within the molecular weight range of 31,000-34,000 daltons (see Figure 10A).

Example 6

Determination of Molecular Weights of Purified Osteogenic Factors Under Reducing Conditions and Purification of Reduced Subunits

Purified osteogenically active proteins in the HPLC Active Pool were subjected to an alternative analytical method whereby protein subunits held together by disulfide bonds can be resolved by reduction of these bonds in SDS dilution buffer in the presence of a reducing agent (dithiothreitol or β-mercaptoethanol) and electrophoreses on 12.5% or 15% SDS polyacrylamide gels. Molecular weights were determined relative to

non-prestained molecular weight standards (Bio-Rad). In this gel system, the HPLC Active Pool revealed proteins migrating as two broad bands within the molecular weight ranges of 16,000-17,500 and 17,500-19,000 daltons (see Figure 10B).

The HPLC Active Pool was made 6 M in guanidine hydrochloride, 50 mM in ethanolamine and 50 mM in dithiothreitol to reduce the disulfide bonds. The reduced sample was diluted at least 2 fold with either water or 0.05% trifluoroacetic acid in water and loaded onto an analytical C-18 HPLC column equilibrated with a buffer comprising, by volume, 70% Buffer A and 30% Buffer B, as described previously (Buffer A is 0.05% trifluoroacetic acid in water and Buffer B is 0.025% trifluoroacetic acid in acetonitrile). Bound proteins were eluted using a linear gradient of 30% to 60% acetonitrile in 60 minutes. Four prominent peaks of protein, designated A, B, C and D, were detected by monitoring UV absorbence at 229 nm; these eluted within the concentrations of 40% to 47% acetonitrile (see Figure 11A). When analyzed by reducing SDS gel electrophoresis followed by silver staining, the reduced subunit A migrated within the molecular weight range of 17,500-19,000 daltons, the reduced subunit B migrated within the molecular weight range of 16,000-17,500, the reduced subunit C migrated within the molecular weight range of 17,500-19,000 and the reduced subunit D migrated within the molecular weight range of 17,500-19,000 (see Figure 11B).

Example 7

Amino Acid Sequences of Bovine Osteogenically Active Proteins P3 OF 31-34

The isolated reduced subunits purified from HPLC Active Pool as disclosed in Example 6, were analyzed by a gas phase sequenator (Applied Biosystems,

Model 470A), and found to have the following amino-terminal sequences:

Subunit A: SAPGRRRQQARNRSTPAQDV

Subunit C: SXKHXXQRXRKKNNN

Subunit D: STGGKQRSQNRSKTPKNQEA

where the amino acids are represented by the well known one-letter and three-letter designations presented in Table 4 below.

Table 4

Amino Acid	Three-Letter Abbreviation	One-Letter Symbol
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic Acid	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid	Glu	E
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V
Undetermined		X

The isolated subunit B yielded no detectable

amino-terminal sequence. When subunit B was digested with Staph V8 protease, and rechromatographed by HPLC, two detectable internal fragments were isolated having the following amino acid sequences:

Subunit B: XVVLKNYQDMV

XEKVVLKNYQDM

where X represents an unassigned amino acid.

Example 8

Osteogenic Compositions for Implantation

The osteogenic preparations of the invention may be used to prepare osteogenic compositions for implantation into mammals. The Prep HPLC Pool of Example 2 may be admixed with one or more of a variety of physiologically acceptable matrices. Such matrices may be resorbable, non-resorbable or partially resorbable. Resorbable matrices include polylactic acid polycaprolactic acid, polyglycolic acid, collagen, plaster of paris and a variety of thermoplastic polymer materials. Non-resorbable materials include hydroxyapatite and partially resorbable materials include matrices such as tricalcium phosphate. The Prep HPLC Active Pool may be adsorbed onto the matrix material which can be either in a granular or solid form. The osteogenic composition may then be dried by lyophilization.

Example 9

Device Coated With Osteogenic Preparations

In this example, the Prep HPLC Active Pool of Example 2 containing the osteogenically active proteins was used to form osteogenically active devices useful for the healing of bone defects. The devices were prepared by absorbing the Prep HPLC Active Pool onto solid delivery matrices comprising either a porous hydroxyapatite disc (Interpore 200, Interpore

International, Irvine, CA) or a porous polylactic acid disc (DRILAC, OSMED Incorporated, Costa Mesa, CA). The discs were 8 to 10 mm in diameter and 3 mm thick and were coated with 0.2 to 0.3 mg of the Prep HPLC Active Pool which was dried onto the matrices by lyophilization. The device may then be sterilized by gamma-irradiation with as much as 3.3 to 3.5 M rads or other suitable means. The devices comprising the osteogenic preparation and the matrix were implanted into trephine defects created in New Zealand Albino Female rabbits, weighing 2.5 to 3.0 kg. Specifically, test devices either coated with the osteogenic preparation or not coated with the osteogenic preparation were surgically implanted into the calvaria using appropriate aseptic surgical techniques. Animals were anesthetized with an intramuscular injection of Ketamine and Xylazine. Following a midline incision, the calvarium was exposed and two trephine holes (one on each side of the midline) 5 mm posterior to the orbits, 8-10 mm in diameter and to the depth of the dura were cut into the calvarium. Trephine defects were created using a Stille cranial drill, exercising great care not to injure the dura. A test device was implanted into one trephine hole while the trephine hole on the opposite side was left empty. Following surgical implantation, antibiotic prophylaxis with penicillin and streptomycin was administered. The animals were followed daily by clinical observations. At explant, the calvaria was removed en block. The specimens were fixed in 10% buffered formalin, decalcified and processed for hematoxylin and eosin stained sections. Histomorphology and qualitative determination of percent ossification was determined by examination of the stained sections (see Table 5 below). The percent area of activity is estimated by eye from the fields of view, or fraction of fields of view, of newly formed bone

matrix as compared to the total fields of view not occupied by the matrix in the entire full cross section.

Table 5

% Ossification in Devices Implanted into Rabbit Trephine Defects

Time of Explant / Test Device	6 weeks	12 weeks
Uncoated Hydroxyapatite	<10%	<25%
Uncoated Polylactic Acid	<10%	<10%
Hydroxyapatite Coated with Osteogenic Preparation	>90%	>90%
Polylactic Acid Coated with Osteogenic Preparation	>90%	>90%
Hydroxyapatite Coated with Osteogenic Preparation and Treated with Gamma-Irradiation	>75%	>90%

Example 10

Amino Acid Sequences of CNBr Fragments of P3 OF 31-34

The isolated reduced subunits purified from HPLC Active Pool (Example 6) were adsorbed onto polyvinylidine difloride (PVDF) transfer membrane (Millipore, Bedford, MA), exposed to vapors from 80 mg/ml CNBr in 70% formic acid for 15 to 20 hours and sequenced using the gas phase sequenator. The following amino acid sequences are represented by the well-known one-letter designations presented in Table 4.

Subunit A, following cleavage with CNBr, yielded sequences from the simultaneous sequencing of several fragments corresponding to the amino terminal sequence described in Example 7:

ANt: SAPGRRRQQARNRSTPAQDV

and an internal fragment:

A1: NPEYVPK

Subunit B, following cleavage with CNBr, yielded sequences from the simultaneous sequencing of two internal fragments:

B1: LYLDENEK

B2: VVEGXGXR

When compared with the sequences of fragments of subunit B cleaved with staph V8 protease (Example 7), fragments B1 and B2 contain overlapping regions, allowing an extended internal sequence in subunit B:

```
B1:          LYLDENEK
Staph V8:          XEKVVLKNYQDM
Staph V8:            XVVLKNYQDMV
B2:                          VVEGXGXR
Consensus:   LYLDENEKVVLKNYQDMVVEGXGXR
```

Subunit D, following cleavage with CNBr, yielded sequences from the simultaneous sequencing of several fragments corresponding to the amino terminal sequence described in Example 7:

DNt: STGGKQRSQNRSKTPKNQEA

and an internal sequence:

D1: XATNHAIVQTLVHFIN

The isolated reduced subunit C, purified from the HPLC Active Pool (Example 6), was adsorbed onto a PVDF transfer membrane, subjected to 20 cycles of amino terminal sequencing using the gas phase sequenator, subjected to cleavage by CNBr vapors, and then sequenced using the gas phase sequenator. Subunit C, following cleavage with CNBr, yielded the following internal sequence:

C1: LYLXEYDXVVLXNYQ

The amino terminal and internal sequences of subunits A, B, C and D derived from bovine bone can be aligned with homologous regions from the deduced amino acid sequences of cDNA clones encoding the polypeptides designated BMP-2A, BMP-2B and Vgr-1 (Figure 12). Homologous regions for the deduced sequences of BMP-2A,

BMP-2B (Wozney, et al., Science, 242, 1528-1534 (1988)) and Vgr-1 (Lyons, et al., Proc. Natl. Acad. Sci. USA, 86, 4554-4558 (1989)) are boxed. Homologous residues in the sequences for bovine subunits A, B, C and D, as compared to the deduced sequences for BMP-2A, BMP-2B and Vgr-1, are bold-faced. Comparison of the similarities and differences of the sequences of subunits B and C and the sequences of BMP-2A and BMP-2B indicate that bovine subunit B shares the same sequence as BMP-2A while bovine subunit C shares the same sequence as BMP-2B.

Example 11

Subunit Compositions of Purified Osteogenically Active Proteins P3 OF 31-34

Individual fractions, eluting within the HPLC active pool (Example 5) and containing the osteogenically active proteins P3 OF 31-34 (Figure 13A), were analyzed by SDS polyacrylamide gel electrophoresis in the absence of reducing reagents (Figure 13B). Figure 13A shows the elution profile obtained by high performance liquid chromatography, on a reverse phase C18 column of the PS Pool. Figure 13B shows non-reducing SDS polyacrylamide gel electrophoresis of P3 OF 31-34 proteins eluting in fractions 26, 27 and 28 from the reverse phase HPLC of the PS Pool. These individual fractions were further analyzed (as described in Example 6) by reduction of the disulfide bonds with 50 mM dithiothreitol in 50 mM ethanolamine and 6 M guanidine hydrochloride and chromatography on a C18 HPLC column (Figure 14). Figure 14A shows the isolation and identification of subunits of the P3 OF 31-34 proteins eluting in fraction 26 from the reverse phase HPLC of the PS Pool, while Figure 14B shows the isolation and identification of P3 OF 31-34 proteins eluting in fraction 28. Subunits A, B, C and D are designated by the solid lines in the figures. Fraction 26, the sample

comprising the lowermost band of the P3 OF 31-34 region (Band I of Figure 13B), was found to contain predominantly subunits B and D with smaller amounts of subunits A and C. Fraction 28, the sample comprising predominantly the uppermost band of the P3 OF 31-34 region (Band II of Figure 13B), together with a smaller amount of Band I, was found to contain increased amounts of subunits A and C, and a decreased amount of subunit D, as compared to the relative amount of subunit B.

These individual fractions, eluting within the HPLC active pool and containing the osteogenically active proteins P3 OF 31-34, were electrophoresed on 12.5% SDS polyacrylamide gels in the absence of reducing reagent (-DTT), electrophoretically transferred to polyvinylidine difloride (PVDF) transfer membranes in the presence of 10% methanol, 10 mM cyclohexylamino-1-propanesulfonic acid, pH 10-11, at 0.5 amp for 15 to 30 minutes, and visualized by staining with Coomassie brilliant blue R250. Individual protein bands in the region of P3 OF 31-34, defined here as Band I (lower) and Band II (upper), were sliced from the membrane and subjected first to N-terminal sequencing, and then to internal sequencing following treatment with CNBr as described in Example 10. These procedures revealed the following sequence for Bands I and II:

Band Sequenced		Sequences	Subunit Identity
Band I	Internal	XATNXAIVQTL	D
		LYLDEXEXVVL	B
Band II	N-Terminal	XXXGRRXQ	A
		XXGGXQR	D
Band II	Internal	LYLDXNXXVVLXN	B
		XPEXVPX	A

where the amino acids are represented by the well-known one-letter designations presented in Table 4.

These results indicated that Band I, the lowermost band of the P3 OF 31-34 proteins, contains predominantly subunits D and B, and that Band II, the uppermost band of the P3 OF 31-34 proteins, contains predominantly subunits A and B. These compositions, as well as the observation that these subunits are purified as disulfide-linked dimers in the purified P3 OF 31-34 proteins (Examples 5 and 6), indicate that subunits A and B may be disulfide-linked as a heterodimer, and that subunits D and B may be disulfide-linked as another heterodimer.

Example 12

Polyclonal Antisera Against Osteogenically Active Proteins P3 OF 31-34

Antisera specific for proteins containing subunits A or D were generated against the following synthetic peptides obtained from Peninsula Laboratories, Belmont, California:

Antigen		Antibody Designation
Subunit A	$(SAPGRRRQQARNRSTPAQDV)_8lys_7$	AbANt
Subunit D	$(STGGKRRSQNRSKTPKNQEA)_8lys_7$	AbDNt

Antisera were generated in rabbits (3- to 6-month-old New Zealand white male) using standard procedures of subcutaneous injections, first in complete Freunds adjuvant, and later (at 14 and 21 days) in incomplete Freunds adjuvant followed by bleeding and preparation of antisera.

The AbANt and AbDNt antisera were cross-reactive with the synthetic peptide antigens when used in an ELISA or Dot Blot format and the reduced subunits A and D when used in a Western Blot format. The AbANt and AbDNt antisera were also cross-reactive with the

osteogenically active proteins P3 OF 31-34 when used in either an ELISA, Western or Dot Blot format. These antisera are not cross-reactive with any presently defined form of subunit B or subunit C as determined by Western Blot and Dot Blot analysis as against purified subunit B and subunit C.

Example 13

Presence of P3 OF 31-34 Proteins in P3

The protein contained in P3 was isolated substantially as described in Example 1 and Figures 2 and 3, and was purified utilizing gel filtration on Sepharose S-200 and reverse-phase HPLC on a Protesil 300 octyl column equilibrated in 0.1% TFA and 10% ACN. The protein was then suspended in a SDS dilution buffer in the presence of reducing agents (+DTT) and electrophoresed on 12.5 or 15% SDS polyacrylamide gels (SDS-PAGE). Proteins contained within the gel were visualized using Coomassie brilliant blue, or were electrophoretically transferred to nitrocellulose in the presence of 10% methanol, 10 mM cyclohexylamino-1-propanesulfonic acid (CAPS), pH 10-11, at 0.5 amp for 15 to 30 minutes. The nitrocellulose filter was treated for Western Blot analysis utilizing antibodies generated against synthetic peptides of the N terminal sequences of subunit A (AbANt) and subunit D (AbDNt).

The nitrocellulose paper containing the protein was placed in a solution-designated buffer P (composed of 20 mM phosphate, pH 7.4; 0.15 M NaCl; 0.05% Tween-20; 0.25% gelatin; and 0.02% sodium azide) for a minimum of 1 hour at 22°C with agitation.

Buffer P was then replaced by buffer Q (composed of buffer P plus antibodies AbANt and AbDNt) for a minimum of one hour at 22°C (or overnight at 4°C). Buffer Q was replaced by Buffer P, which was changed four times over a minimum of one hour. Buffer P

was replaced by Buffer R (buffer P plus ^{125}I protein A at 2.5 x 10^5 cpm/ml, Amersham) and incubated for one hour at 22°C with agitation. Buffer R was replaced by Buffer P, which was changed at least four times during one hour of incubation.

The moist nitrocellulose filter was placed between sheets of plastic wrap, and together with a lighting screen and X-ray film (Dupont Cronex, Wilmington, DE), enclosed in a light-proof folder, and placed at -70°C for an appropriate period of time. The exposed film was developed using standard techniques and equipment, and the resulting autoradiograph shown in Figure 15 demonstrates the presence of subunits A and/or D within the P3 fraction.

Example 14

Glycosylation of Bovine Osteogenically Active Protein P3 OF 31-34

Reduced subunits A and D were purified from HPLC active pool as disclosed in Example 6 and were subjected to digestion by Peptide-N^4 (N-acetyl-beta-glucosaminyl) arginine amidase (N-glycanase, Genzyme) and endo-beta-N-acetyl glucosaminidase H (endo H, Genzyme) according to manufacturer's specifications. The relative molecular weights of the reduced subunits, both before and after digestion with the endoglycosidases, was evaluated by electrophoresis on a 15% SDS polyacrylamide gel, followed by Western analysis using the antibodies designated as AbANt and AbDNt. Figure 16 shows reducing SDS polyacrylamide gel electrophoresis of reduced subunits A and D before and after treatment with either endo H or N-glycanase. Western Blot analysis of isolated reduced subunit A, both before and after treatment with glycosidases, is shown in panel A. Western Blot analysis of isolated reduced subunit D, both before and after treatment with

glycosidases, is shown in panel D. A decrease in the relative molecular weights, from approximately 17,500-19,000 daltons to 14,000-16,000 daltons, of each of the digested subunits A and D, indicated that the subunits A and D contain asparagine-linked carbohydrate which was sensitive to digestion either by endo H or N-glycanase.

Example 15

Identification of Sequences of Human cDNA Encoding Proteins Homologous to Subunit D of Bovine Osteogenically Active Proteins P3 OF 31-34

A variety of techniques can be used to identify sequences of human DNA encoding proteins homologous to a particular sequenced protein. Such methods include the screening of human DNA, human genomic libraries and human cDNA libraries. A variety of oligonucleotide probes can be used including probes exactly complementary to the human DNA sequence, mixtures of probes complementary to all or some of the possible DNA sequences coding for the particular protein sequence, degenerate probes synthesized such that all possible sequences complementary to all possible DNA sequences coding for the particular protein sequence are represented, and degenerate probes synthesized using nucleotide analogues such as deoxyinosine triphosphate. In this example, the polymerase chain reaction (PCR) technique was used to amplify sequences of human cDNA encoding proteins homologous to subunit D of bovine osteogenically active proteins P3 OF 31-34.

Preparation of cDNA from U-2 OS Cells

The human osteogenic sarcoma cell line U-2 OS was obtained from the ATCC (American Type Culture Collection, Rockville, MD) and maintained in McCoy's 5a medium supplemented with 10% fetal calf serum and 1% glutamine/penicillin/streptomycin. Unless otherwise

described, DNA manipulations, definition of terms, and compositions of buffers and solutions are described by Maniatis, T., et al., Molecular Cloning: A Laboratory Manual (1982). Poly $(A)^+$ RNA was isolated from U-2 OS cells using the Fast Track-mRNA isolation kit from Invitrogen (San Diego, CA). A first strand cDNA copy of the mRNA was generated with oligo (dT) as the primer using the AMV Reverse Transcriptase System I from Bethesda Research Laboratories (BRL, Gaithersburg, MD). Each reaction used 1 µg of poly $(A)^+$ RNA which was reverse transcribed into first strand cDNA that was used as template in eight separate polymerase chain reaction (PCR) DNA amplification reactions. Following cDNA synthesis, RNA was hydrolyzed by treatment with 50 mM NaOH at 65°C, followed by neutralization in 0.2 N HCl.

PCR Amplification

Polymerase chain reaction (PCR), as described by R.K. Saiki, et al., Science 239:487-491 (1988), was used to amplify DNA from U-2 OS cDNA prepared as described above. Oligonucleotide primers for PCR were synthesized on an automated DNA synthesizer and were derived from the amino terminal and internal amino acid sequences of bovine subunit D. The 5' PCR primer, designated ODM-1, corresponded to sequence from the first 11 amino acids from the amino terminus of bovine subunit D, namely STGGKQRSQNR. This 32-mer contained all possible combinations of nucleotide sequence coding for this sequence of amino acids and was greater than 4-million-fold degenerate. The nucleotide sequence of ODM-1 was 5'-[T/A][C/G]NACNGGNGGNAA[G/A]CA[G/A][C/A]GN [T/A][C/G]NCA[G/A]AA[C/T][C/A]G-3'. Bracketed nucleotides are alternatives, and "N" means all alternatives (A, C, T and G).

The 3' PCR primer corresponded to an internal sequence of bovine subunit D, namely, NHAIVQTLVHFIN, and

was synthesized as the inverse and complementary sequence. This oligonucleotide primer was designated ODB-1 and had the sequence 5'-TTTTTTTTGGATCC[G/A]TTXAT [G/A]AA[G/A]TGXACXA[G/A]XGT[C/T]TGXACXATXGC[G/A]TG[G/A]T T-3'. Bracketed nucleotides are alternatives, and "X" represents the nucleotide analog deoxyinosine-triphosphate (dITP), which was used in all positions where all four of the nucleotides (A, C, T or G) were possible. The sequence is preceded on the 5' end by a string of eight T's, followed by the sequence GGATCC which designates a BamHI recognition site, leaving a stretch of 39 nucleotides corresponding to the internal amino acid sequence of bovine subunit D.

Amplification of DNA sequences coding proteins homologous to bovine subunit D using these two primers was accomplished using the Perkin-Elmer Cetus Gene Amp DNA Amplification Reagent Kit (obtained either from Perkin-Elmer Cetus, Norwalk, CT, or United States Biochemical Corporation, Cleveland, OH). The PCR reaction contained 1 µg of each primer ODM-1 and ODB-1, 1/8 of the synthesized U-2 OS first strand cDNA (approximately 25-50 ng), 200 µM of each dNTP, and 2.5U Ampli-Taq DNA Polymerase in the kit-supplied reaction buffer of 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% (w/v) gelatin. PCR was performed for 30 cycles consisting of 1.5 minutes denaturation at 94°C, 2 minutes annealing at 50°C and 3 minutes elongation at 72°C. After the 30 cycles, a final 10-minute elongation at 72°C is performed.

The PCR products were analyzed by agarose gel electrophoresis, which revealed a major band of amplified DNA of approximately 300 bp. A Southern Blot was performed in which the DNA in the gel was transferred to a Nytran nylon membrane (Schleicher and Schuell, Keene, NH) using an LKB Vacugene Vacuum Blotting Unit, and then the DNA was UV-crosslinked to

the membrane using a Stratalinker (Stratagene, La Jolla, CA). The membrane was probed for amplified sequences encoding proteins homologous to bovine subunit D using a probe corresponding to the amino acid sequence KTPKNQEALR. This sequence is found near the amino terminus of bovine subunit D, following the sequence used to constructed the 5' PCR primer. This probe would therefore hybridize to amplified sequences that encode proteins homologous to bovine subunit D without overlapping either of the two primers used in the amplification. This 29-mer probe was designated ODibb and had the sequence AAXACXCCXAA[G/A]AA[C/T]CAXGA[G/A] GCX[C/T]TX[C/A]G, where bracketed nucleotides are alternative and "X" represents dITP, which was used in positions where all four nucleotides (A, C, T or G) were possible. The Southern Blot was prehybridized at 42°C in 5xSSPE, 0.5% SDS, 3x Denhardt's, 100 µg/ml salmon sperm DNA, then hybridized at 42°C in 6xSSPE, 0.5% SDS to the ODibb probe which had been radioactively labelled using polynucleotide kinase and $\gamma[^{32}P]$ATP. The blot was washed at 42°C in 2xSSC, 0.1% SDS. Autoradiography of the blot showed that ODibb hybridized specifically to the 300-bp PCR-amplified DNA.

Example 16

Cloning and Sequencing of Human cDNA's Encoding Proteins Homologous to Subunit D of Bovine Osteogenically Active Proteins P3 OF 31-34

5' phosphates were added to the blunt-ended PCR product of Example 15 using kinase and ATP, and the DNA was then ligated into the SmaI cut (blunt end) site of the vector pT7T3 18U (Pharmacia, Piscataway, NJ). Following digestion with SmaI to linearize any religated vector, the recombinant plasmid DNA was used to transform E. coli TG1 cells. Several transformants were picked and used to purify plasmid DNA by a mini-lysate procedure. The size of the insert contained in these

plasmids was confirmed to be 300 bp by restriction analysis.

DNAs from seven different transformants were sequenced by dideoxy sequencing methods (Sequenase, United States Biochemical Corp). The sequences of three of these clones were identical to each other and, when translated to amino acid sequence, it was confirmed that they were homologous to the sequence of bovine subunit D. The sequence of the PCR-amplified DNA, designated "hOD," is shown in Figure 17, along with the known and derived amino acid sequences. Only the DNA sequence between the two primers is shown, since the degeneracy of the primers did not allow the identification of the exact sequence in these regions. The sequence of the first 34 amplified nucleotides following the ODM-1 primer codes for amino acid sequence previously identified in bovine subunit D.

The sequences of the other four recombinant clones, while identical to each other, were different from the hOD-amplified sequence and encoded a different sequence of amino acids. This family of clones was designated "hOE," and its sequence is shown in Figure 18. Figure 19 shows the homology between hOD and hOE-amplified sequences, indicating 69-70% identity at both the nucleotide and amino acid level in this region. Figure 19A shows the homology between the derived amino acid sequences of the PCR-amplified hOD and hOE sequences wherein the homologous residues are bold-faced. In the region following the first cysteine residue, these two sequences share 39/44 identical amino acids, a highly conserved region among the members of the TGF-β family. Figure 19B shows the homology between the nucleotide sequences of the PCR-amplified sequences designated hOD and hOE.

Example 17

Recombinant Expression of P3 OF 31-34 Subunits

Complete and partial P3 OF 31-34 subunit polypeptide products and analogs may be prepared utilizing recombinant DNA molecules in bacteria, yeast or mammalian expression systems. DNA encoding products based on amino acid sequences derived from isolated P3 OF 31-34 subunits according to the present invention can be inserted into an expression vector, for example, a plasmid, phage or viral expression vector [Vieira, et al., Gene, 19, 259-268 (1982); Young, et al., Proc. Natl. Acad. Sci. (USA), 80, 1194-1198 (1983); Bitter, et al., Gene, 32, 263-274 (1984); Cepko, et al., Cell, 37, 1053-1062 (1984); and Gorman, et al., Mol. Cell. Biol., 2, 1044-1051 (1982)].

In particular, P3 OF 31-34 subunits D and E may be produced by expression of DNA characterized by nucleotide sequences set out in Figures 17 and 18, respectively. Alternatively, DNA characterized by nucleotide sequences encoding the same sequence of amino acids as set out in Figures 17 and 18 could be inserted into an expression vector for the same purpose. Analogs of these subunits could also be prepared by means of DNA sequences which hybridize (or which would hybridize but for the redundancy of the genetic code) with at least 80% of the nucleotide sequence shown in Figures 17 and 18.

Another aspect of applicants' invention involves the preparation of osteogenic materials comprising dimers of subunit D and heterodimers of subunit D and subunit B. Osteogenically active dimers can be produced either by expression of nucleic acid sequences encoding subunit D and subunit B in the same cell allowing disulfide bonds to form during the biosynthetic process, or by separately expressing each subunit in different cells and then combining each

expressed subunit in such a way as to form a disulfide linked dimer. Dimers of subunit A and heterodimers of subunit A and subunit B could similarly be prepared. Osteogenic preparations comprising recombinant produced dimers and heterodimers could be prepared which would have the same osteogenic activity as the P3 OF 31-34 osteogenic preparation isolated according to applicants' methods. Similarly, heterodimers, in which a polypeptide highly homologous to subunit B, such as subunit C, is substituted for subunit B, could also be produced. Pharmaceutically acceptable compositions comprised of such recombinant produced polypeptides in conjunction with physiologically acceptable matrix materials may be prepared and used in the same manner as with polypeptides isolated from human bone.

Numerous modifications and variations in the practice of the invention are expected to occur to those skilled in the art upon consideration of the foregoing descriptions of preferred embodiments thereof. Consequently, only such limitations should be placed upon the invention as appear in the following claims.

WE CLAIM:

1. A preparation of an osteogenic protein characterized by a molecular weight of from about 25,000 to about 38,000 daltons as characterized by non-reducing denaturing gel filtration and further by the characteristic of eluting at concentrations of between 35% and 45% acetonitrile from a reverse phase high performance liquid chromatography column equilibrated with buffers containing water, acetonitrile and between 0.025% and 0.05% trifluoroacetic acid.

2. The preparation according to claim 1 wherein the osteogenic protein is characterized by a molecular weight of from about 31,000 daltons to about 34,000 daltons as characterized by non-reducing sodium dodecyl sulfate polyacrylamide gel electrophoresis.

3. The preparation according to claims 1 or 2 further characterized by having in its reduced state at least one protein subunit which comigrates on reducing sodium dodecyl sulfate polyacrylamide gels with proteins within the molecular weight range of 17,500 to 19,000 or the range of 16,000 to 17,500 daltons.

4. The preparation according to claim 3 wherein one of said subunits is characterized by an amino-terminal sequence selected from the group consisting of

SAPGRRRQQARNRSTPAQDV,

SXKHXXQRXRKKNNN and

STGGKQRSQNRSKTPKNQEA

or is characterized by an internal amino acid sequence selected from the group consisting of

XVVLKNYQDMV,
XEKVVLKNYQDM,
NPEYVPK,
LYLDENEK,
VVEGXGXR,
XATNHAIVQTLVHFIN and
LYLXEYDXVVLXNYQ

wherein X represents an undetermined amino acid.

5. The preparation according to claim 4 wherein said osteogenic protein comprises a dimer of two subunits linked by at least one disulfide bond.

6. The preparation according to claim 5 wherein said dimer is a heterodimer of two non-identical subunits linked to each other by at least one disulfide bond.

7. The preparation of claim 6 wherein one subunit of said heterodimer is characterized by the internal sequence of NPEYVPK, and the other subunit is characterized by the internal sequence of LYLXEXXXVVLXNYQ.

8. The preparation of claim 6 wherein one subunit of said heterodimer is characterized by the internal sequence of XATNHAIVQTL, and the other subunit is characterized by the internal sequence of LYLXEXXXVVLXNYQ.

9. The preparation according to claim 3 wherein the subunits selected from the group characterized by an amino-terminal sequence consisting of:

SAPGRRRQQARNRSTPAQDV and

STGGKQRSQNRSKTPKNQEA

contain asparagine-linked carbohydrate.

10. The preparation according to claim 1 which is isolated from bovine bone.

11. The preparation according to claim 1 which is isolated from human bone.

12. The preparation according to claim 1 which is isolated from porcine bone.

13. A method for isolating a preparation of an osteogenic protein from demineralized bone tissue, said method comprising:

(a) treating said demineralized bone tissue under aqueous conditions with a solubilizing agent for said osteogenic protein and thereby extracting the osteogenic factor into solution with said solubilizing agent;

(b) subjecting said solution to size fractionation to recover a concentrated pool of proteins of molecular weight between about 10,000 and about 100,000 daltons;

(c) subjecting said concentrated pool to a first chromatography step to recover an active preparation of proteins from a S-Sepharose column

equilibrated with 6.0 M urea containing 50 mM MES pH 6.5 by eluting the active preparation with 6.0 M urea containing 50 mM MES pH 6.5 and 0.5 M NaCl;

(d) subjecting the active preparation of step (c) to a buffer exchange step;

(e) subjecting the active preparation of step (d) to a second chromatography step to recover an active preparation of proteins from a Q-Sepharose column equilibrated with 6 M urea containing 20 mM ethanolamine pH 9.5 by eluting the active preparation with 6.0 M urea containing 20 mM ethanolamine pH 9.5 and 0.2 M NaCl; and

(f) subjecting the active preparation of step (e) to a third chromatography step to recover an active preparation of proteins from a C-18 high performance liquid chromatography column equilibrated with buffers containing trifluoroacetic acid and acetonitrile by eluting the active preparation at concentrations between 35% and 45% acetonitrile.

14. A method for isolating a preparation of on osteogenic protein according to claim 13 further comprising the steps:

(g) subjecting the active fraction of step (f) to a fourth chromatography step to recover an active preparation of proteins from a chelating-Sepharose column charged with Cu^{2+} and equilibrated with 6 M urea containing 50 mM Tris pH 7.5-8.0, 20 mM ethanolamine and 0.5 M NaCl by eluting the active preparation with 6 M urea containing 50 mM Tris pH 7.4-7.8 and 15 mM imidazole;

(h) subjecting the active fraction of step (g) to a fifth chromatography step to recover an active preparation of proteins from a phenyl-Sepharose column equilibrated with 6 M urea containing 50 mM Tris pH 7.4-7.8 and 25% ammonium sulfate by eluting the active

preparation with 6 M urea containing 50 mM Tris pH 7.4-7.8.

(i) subjecting the active preparation of step (h) to a sixth chromatography step to recover an active preparation of proteins from a C-18 high performance liquid chromatography column equilibrated with buffers containing trifluoroacetic acid and acetonitrile by eluting the active preparation at concentrations between 35% and 45% acetonitrile.

15. A preparation of an osteogenic protein characterized by the capacity of promoting osteogenesis in a mammal and prepared according to the method of claim 13.

16. A preparation of an osteogenic protein characterized by a molecular weight of from about 31,000 to 34,000 daltons as characterized by non-reducing sodium dodecyl sulfate polyacrylamide gel electrophoresis, by the capacity of promoting osteogenesis in a mammal and prepared according to the method of claim 14.

17. A method for inducing bone formation in a mammal comprising administering to said mammal an effective amount of the osteogenic preparation of claims 1, 4, 7, 8, 15 or 16.

18. The method of claim 17 wherein said osteogenic preparation is administered admixed with a physiologically acceptable matrix material.

19. The method of claim 17 wherein said osteogenic preparation is administered in the form of a device comprising a structural material encoated with osteogenic preparation admixed with a physiologically acceptable matrix material.

20. A composition for implantation into a mammal comprising the preparation of osteogenic factor according to claims 1, 4, 7, 8, 15 or 16 admixed with a physiologically acceptable matrix material.

21. The composition according to claim 20 wherein said physiologically acceptable matrix material is selected from the group consisting of tricalcium phosphate, hydroxyapatite, collagen, plaster of paris thermoplastic resins, polylactic acid, polyglycolic acid and polycaprolactic acid.

22. A device for implantation into a mammal comprising a structural member encoated with the composition of claim 20.

23. A purified and isolated nucleic acid comprising a nucleotide sequence selected from the group consisting of:

the nucleotide sequence as shown in Figure 17;

a nucleotide sequence which encodes the same sequence of amino acids as encoded by the nucleotide sequence shown in Figure 17;

a nucleotide sequence which is homologous with 80% of the nucleotides shown in Figure 17 and which encodes a polypeptide having osteogenic activity; and

a nucleotide sequence which would be homologous with 80% of the nucleotides shown in Figure 17 but for the redundancy of the genetic code and which encodes a polypeptide having osteogenic activity.

24. A cell transformed with a nucleic acid as recited in claim 23.

25. A polypeptide comprising a continuous sequence of amino acids encoded by a purified and isolated nucleic acid as recited in claim 23.

26. An osteogenic preparation comprising a dimer of two polypeptide subunits linked by at least one disulfide bond comprising a first polypeptide subunit as recited in claim 25.

27. The osteogenic preparation according to claim 26 wherein said dimer is a heterodimer of two non-identical subunits.

28. The osteogenic preparation of claim 27 wherein one polypeptide subunit is characterized by an internal amino acid sequence selected from the group consisting of:

LYLDENEK,
VVEGXGXR,
XVVLXNYQ,
XEKVVLKNYQDM, and
LYLXEXXXVVLXNYQ.

29. A purified and isolated nucleic acid comprising a nucleotide sequence selected from the group consisting of:

the nucleotide sequence as shown in Figure 18;

a nucleotide sequence which encodes the same sequence of amino acids as encoded by the nucleotide sequence shown in Figure 18;

a nucleotide sequence which is homologous with 80% of the nucleotides shown in Figure 18 and which encodes a polypeptide having osteogenic activity; and

a nucleotide sequence which would be homologous with 80% of the nucleotides shown in Figure 18 but for the redundancy of the genetic code and which encodes a polypeptide having osteogenic activity.

30. A cell transformed with a nucleic acid as recited in claim 29.

31. A polypeptide comprising a continuous sequence of amino acids encoded by a purified and isolated nucleic acid as recited in claim 29.

32. An osteogenic preparation comprising a dimer of two polypeptide subunits linked by at least one disulfide bond comprising a first polypeptide subunit as recited in claim 31.

33. The preparation according to claim 32 wherein said dimer is a heterodimer of two non-identical subunits.

34. The osteogenic preparation of claim 33 wherein one polypeptide subunit is characterized by an internal amino acid sequence selected from the group consisting of:

LYLDENEK,
VVEGXGXR,
XVVLXNYQ,
XEKVVLKNYQDM and
LYLXEXXXVVLXNYQ.

35. A process for the preparation of an osteogenic protein consisting of dimers of polypeptide monomers selected from the group consisting of P3 OF 31-34 subunit A, subunit B, subunit C, subunit D and subunit E, the process comprising the steps of culturing in a suitable culture media one or more cell lines transformed with nucleic acid sequences encoding one or more polypeptides selected from the group consisting of P3 OF 31-34 subunit A, subunit B, subunit C, subunit D and subunit E, forming dimers of said polypeptide monomers by linking them with at least one disulfide bond, and isolating said dimers.

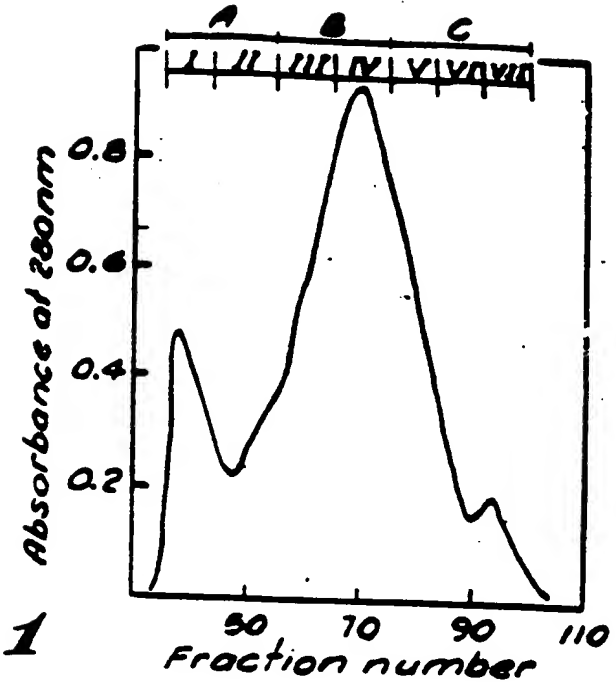

A
B
C
I
II
III
IV
V
VI
VII
VIII
0.8
0.6
0.4
0.2
Absorbance at 280nm
50
70
90
110
Fraction number

Fig. 1

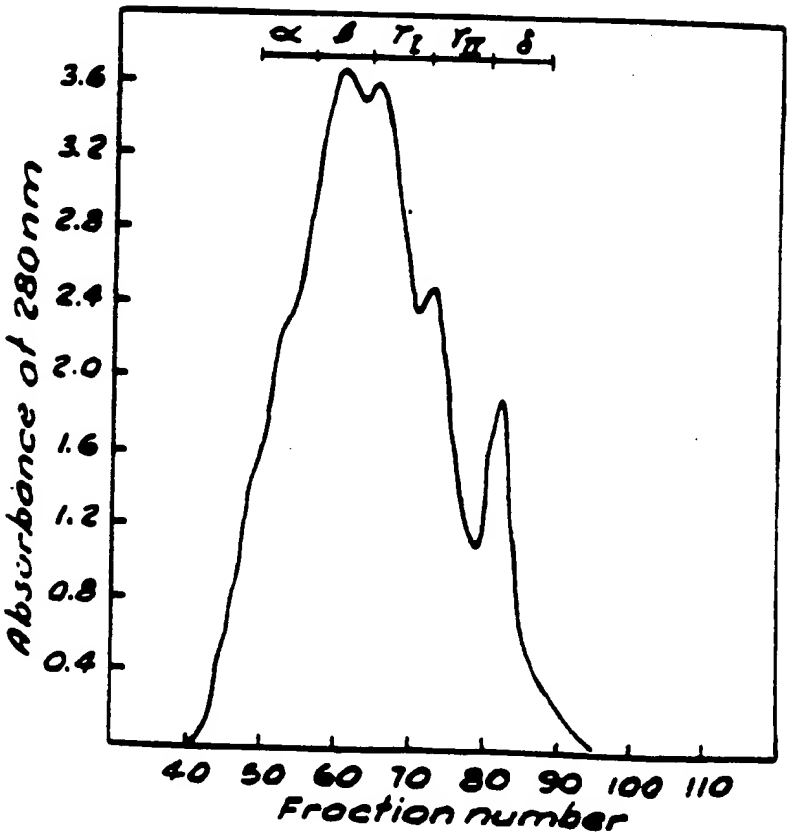

α
β
γ_I
γ_{II}
δ
3.6
3.2
2.8
2.4
2.0
1.6
1.2
0.8
0.4
Absorbance at 280nm
40
50
60
70
80
90
100
110
Fraction number

Fig. 2

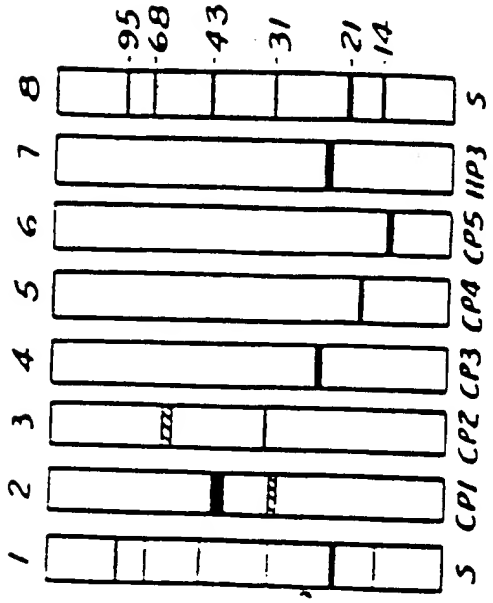

1
2
3
4
5
6
7
8
S
CP1
CP2
CP3
CP4
CP5
IIP3
S
95
68
43
31
21
14

Fig. 4

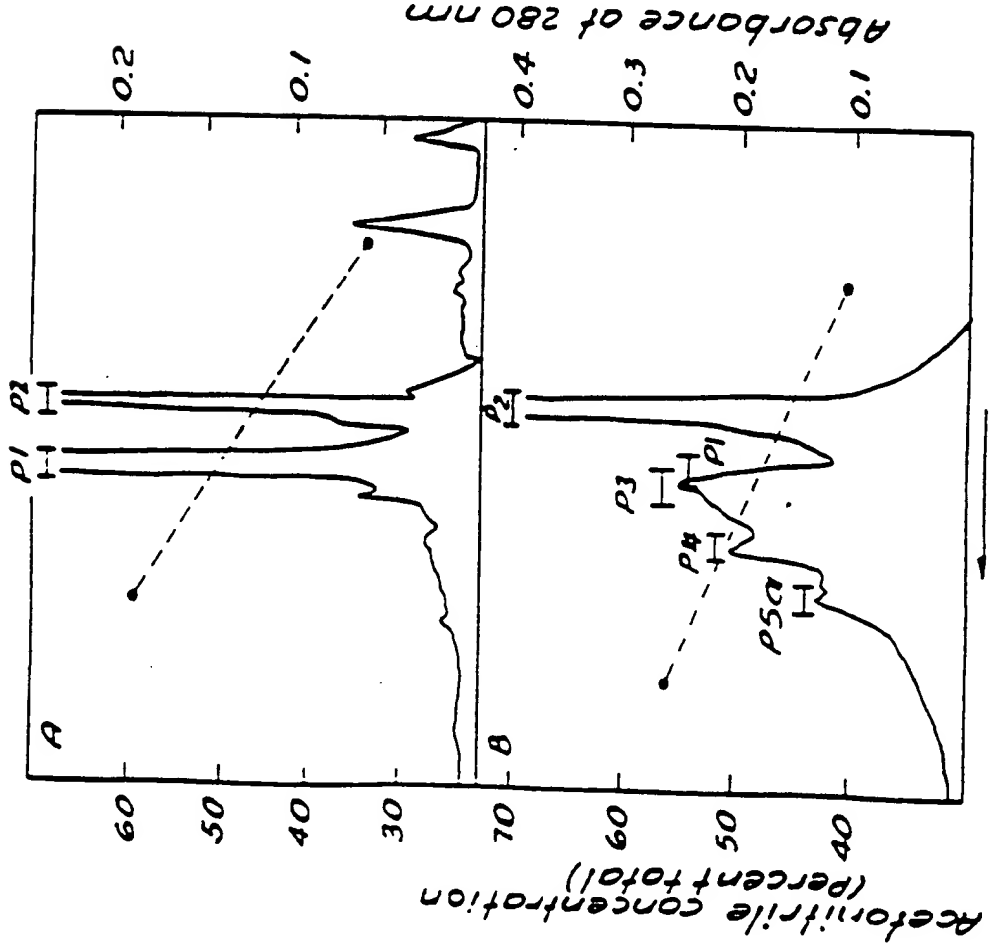

Absorbance of 280 nm
0.2
0.1
0.4
0.3
0.2
0.1
P2
P1
P2
P3
P1
P4
P5a
A
B
60
50
40
30
70
60
50
40
Acetonitrile concentration
(percent total)
Increasing
acetonitrile

Fig. 3

V8 PROTEASE-PORCINE P3 PROTEIN (C8 HPLC COLUMN)

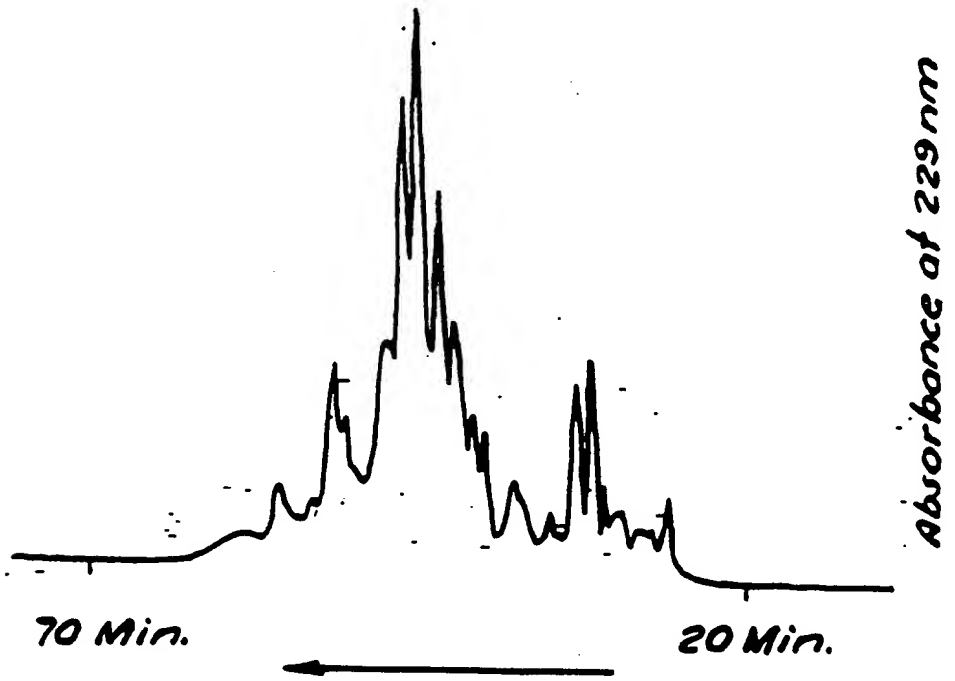


Fig. 5A

V8 PROTEASE-BOVINE P3 PROTEIN (C8 HPLC COLUMN)

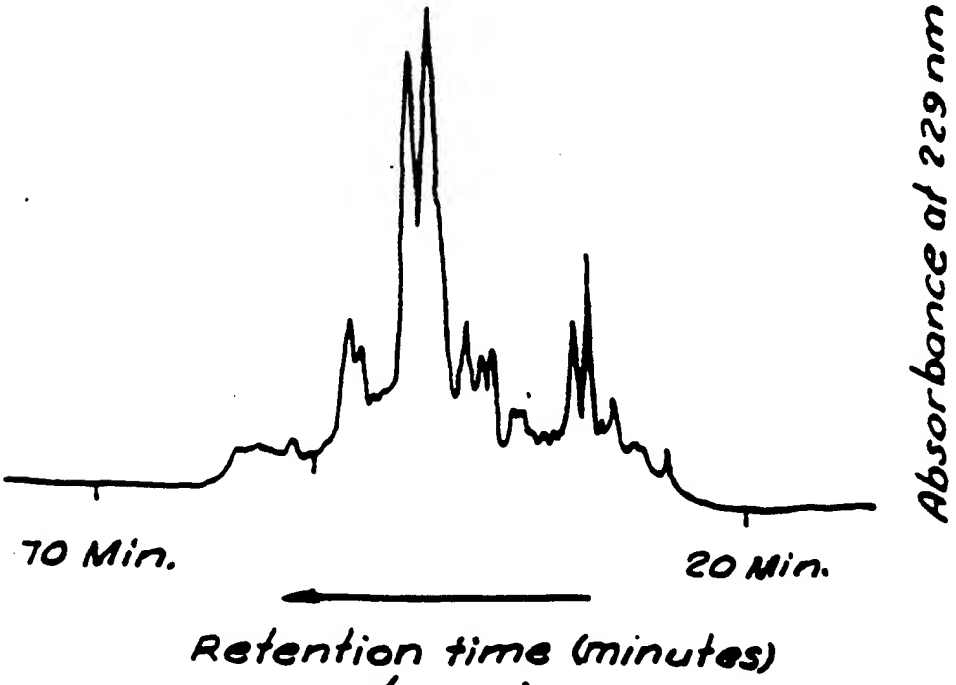


Fig. 5B

V8 PROTEASE - PORCINE P3 PROTEIN (C18 HPLC COLUMN)

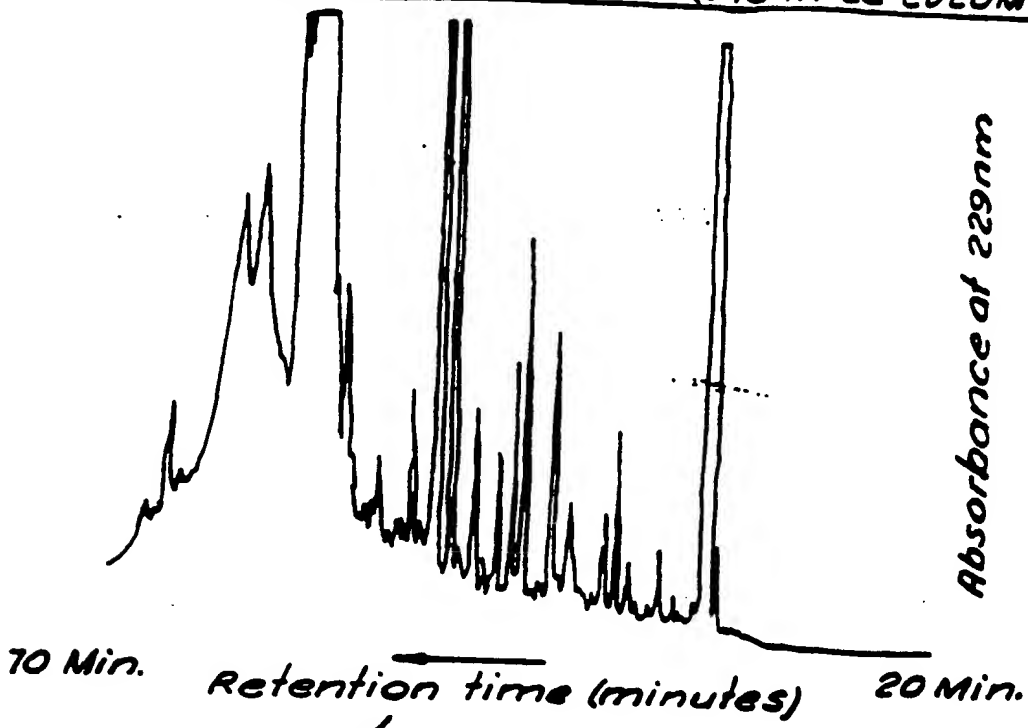


Fig. 6A

V8 PROTEASE - HUMAN P3 PROTEIN (C18 HPLC COLUMN)

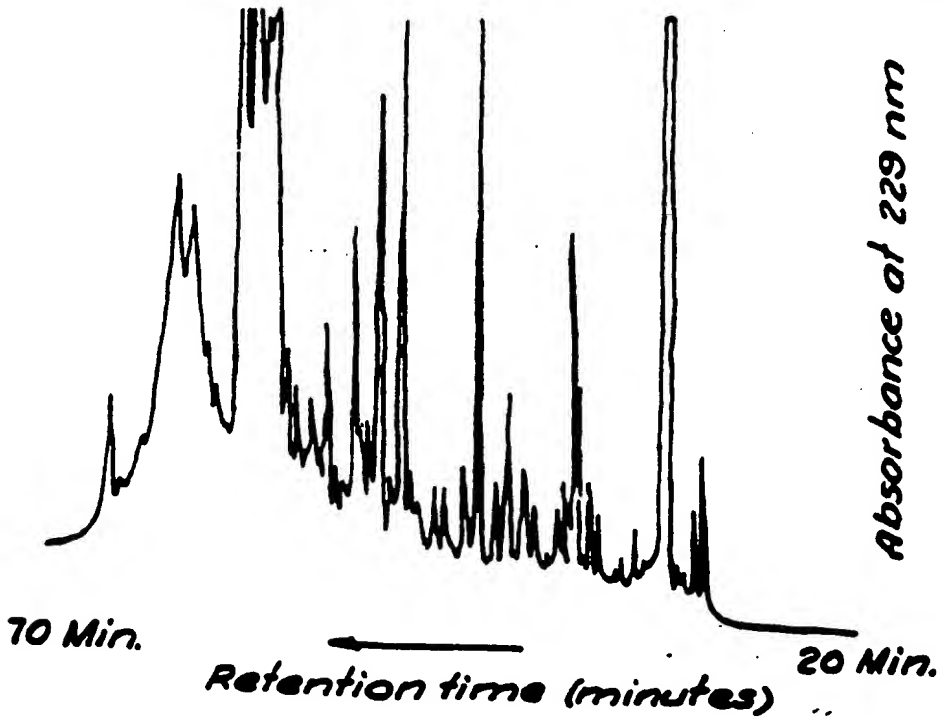


Fig. 6B

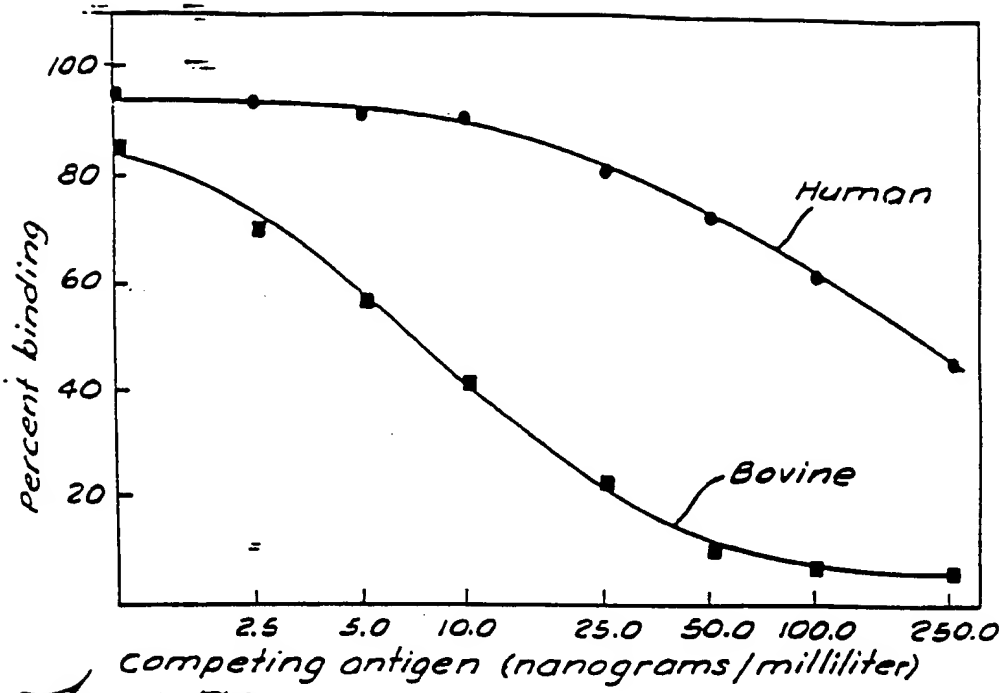

100
80
60
40
20
Percent binding
Human
Bovine
2.5
5.0
10.0
25.0
50.0
100.0
250.0
Competing antigen (nanograms/milliliter)

Fig. 7A

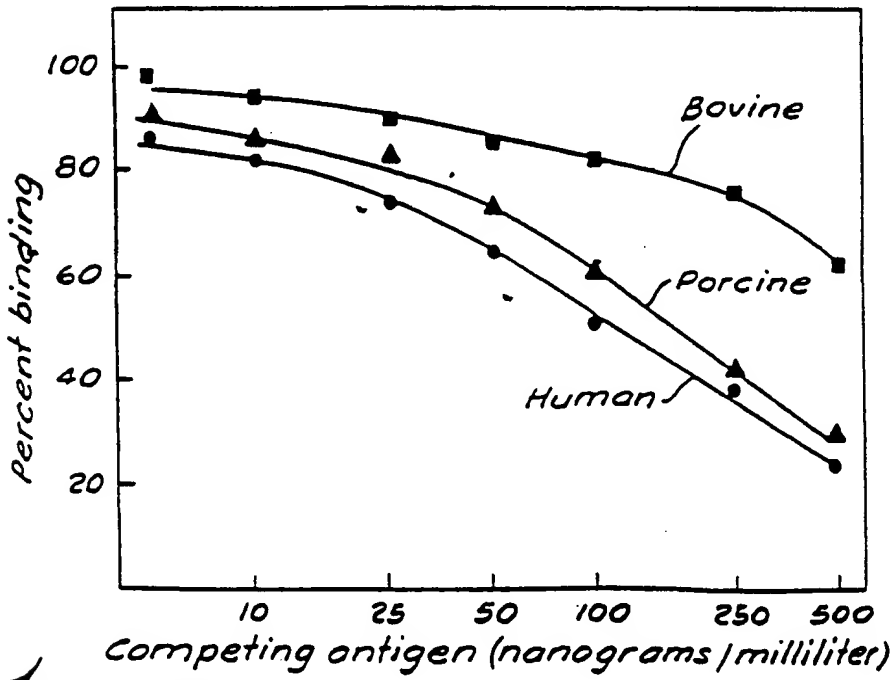

100
80
60
40
20
Percent binding
Bovine
Porcine
Human
10
25
50
100
250
500
Competing antigen (nanograms/milliliter)

Fig. 7B

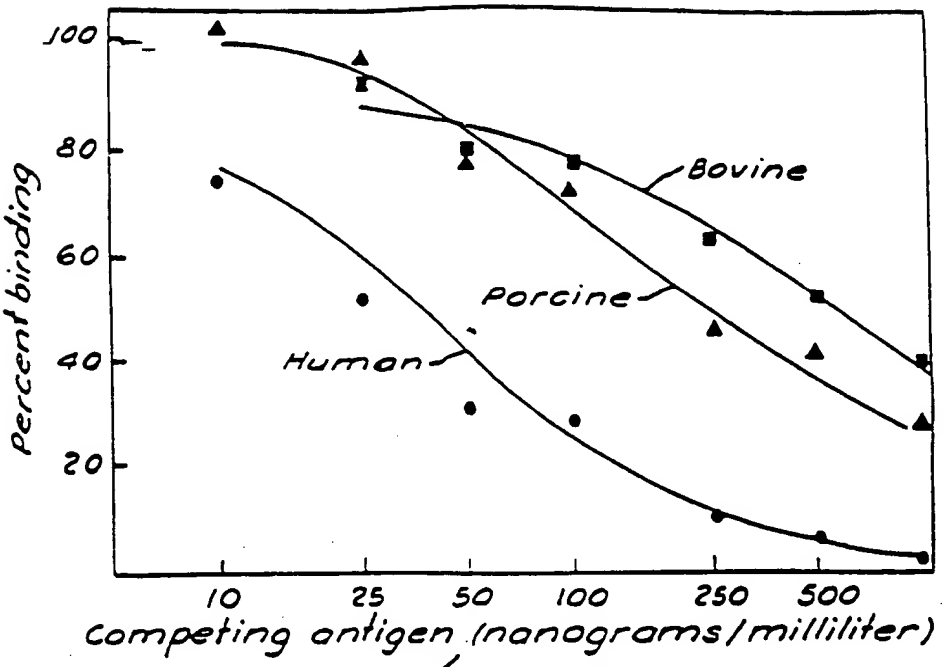

100
80
60
40
20
Percent binding
Bovine
Porcine
Humon
10
25
50
100
250
500
competing antigen (nanograms/milliliter)

Fig. 7C

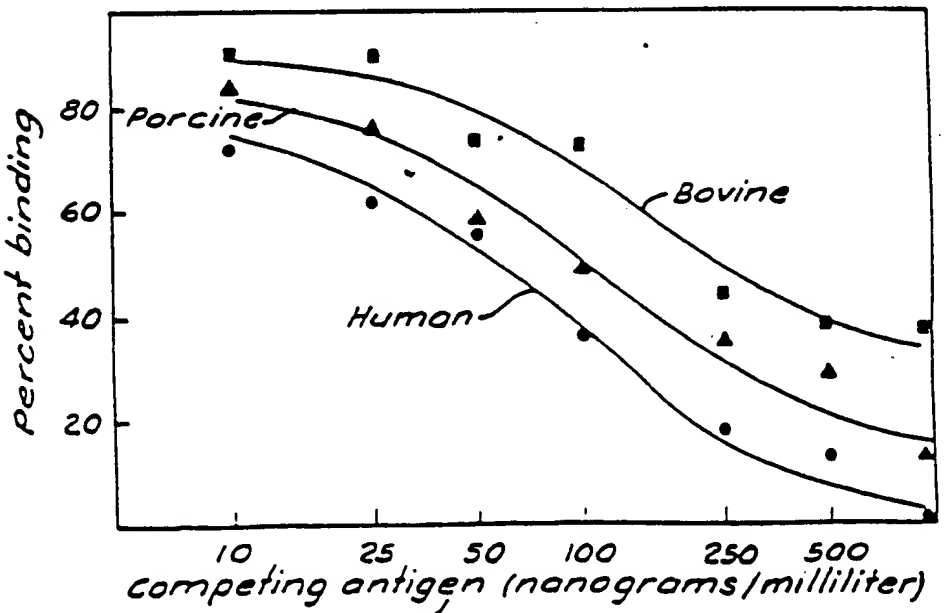
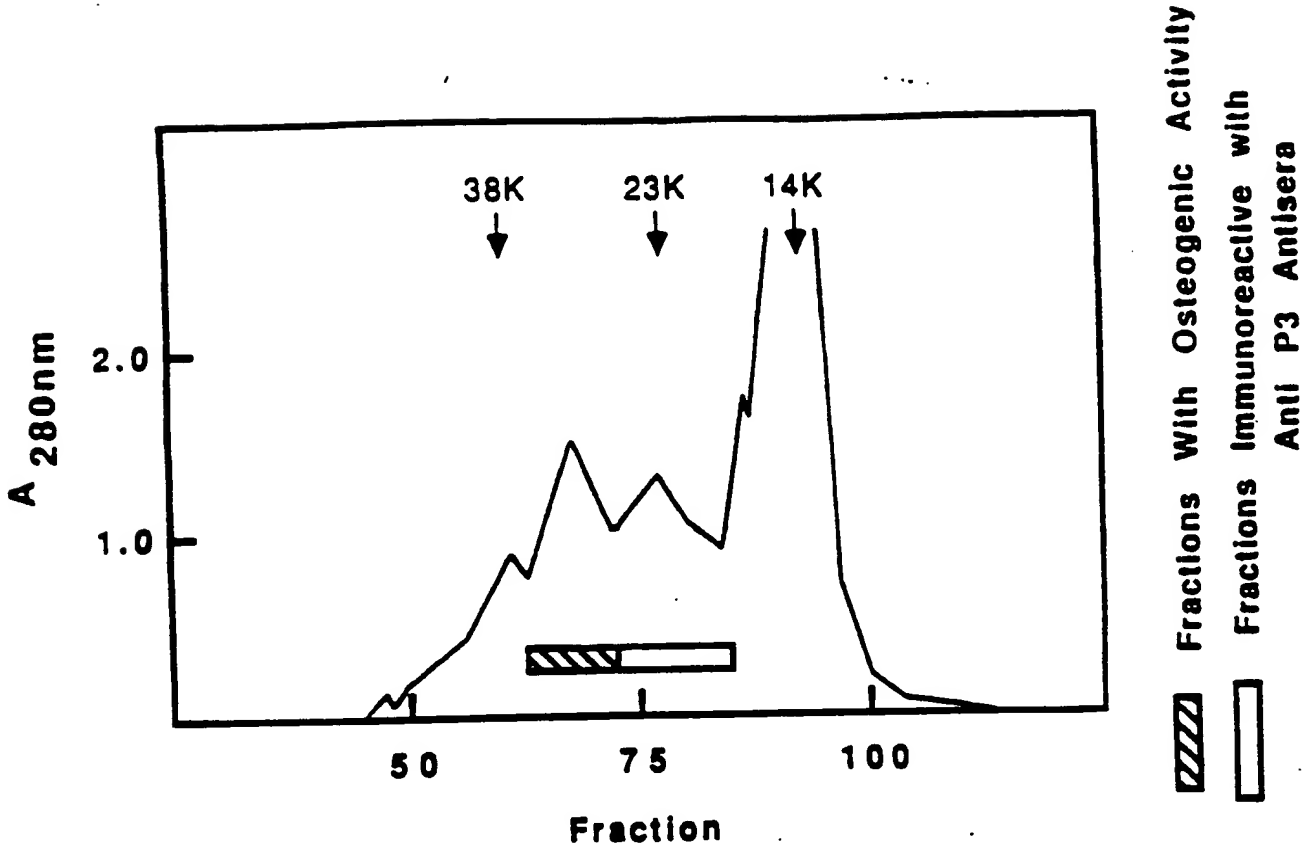

80
60
40
20
Percent binding
Porcine
Bovine
Humon
10
25
50
100
250
500
competing antigen (nanograms/milliliter)

Fig. 7D

Fig. 8


38K
23K
14K
A 280nm
2.0
1.0
50
75
100
Fraction
Fractions With Osteogenic Activity
Fractions Immunoreactive with Anti P3 Antisera

Purification of Osteogenic Factors

Fig. 9

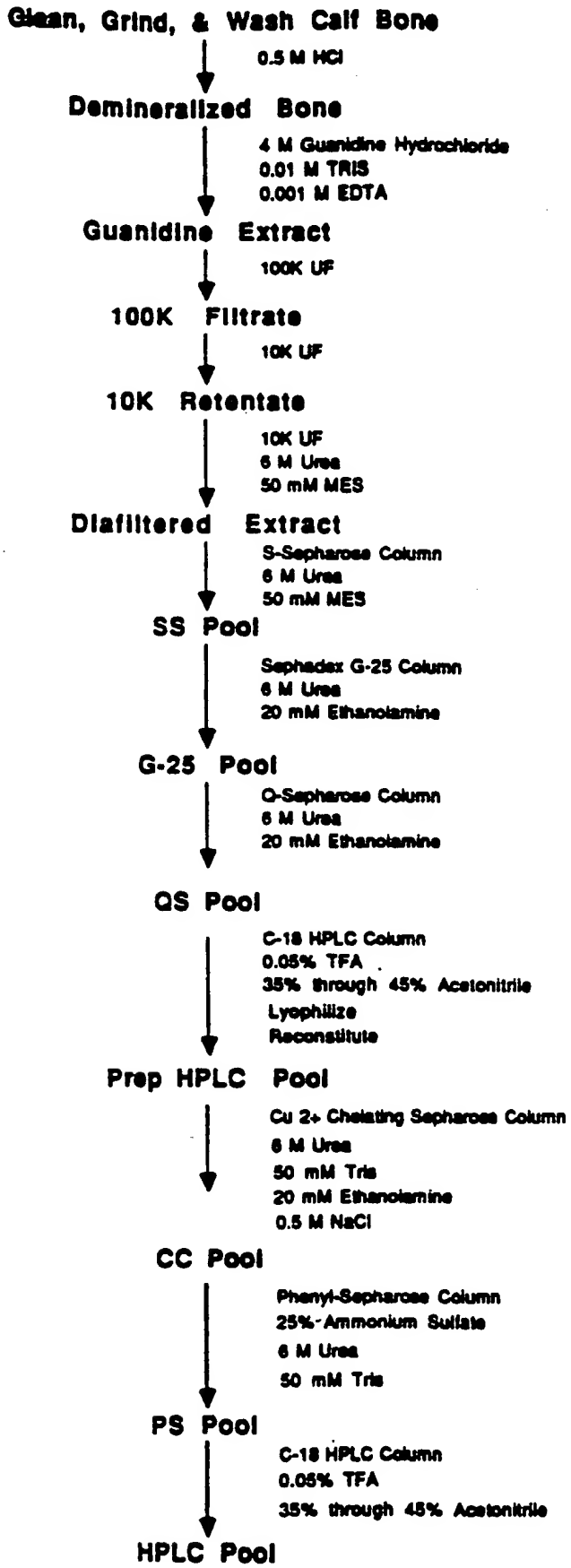


Fig. 10A

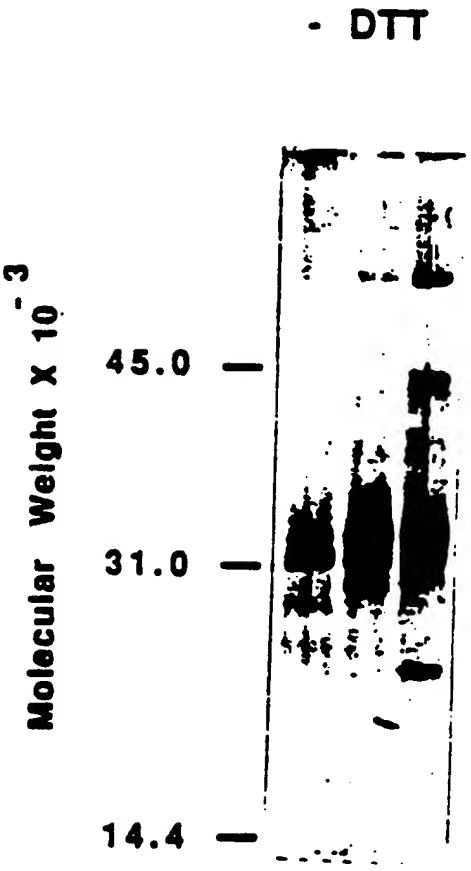


Fig. 10B

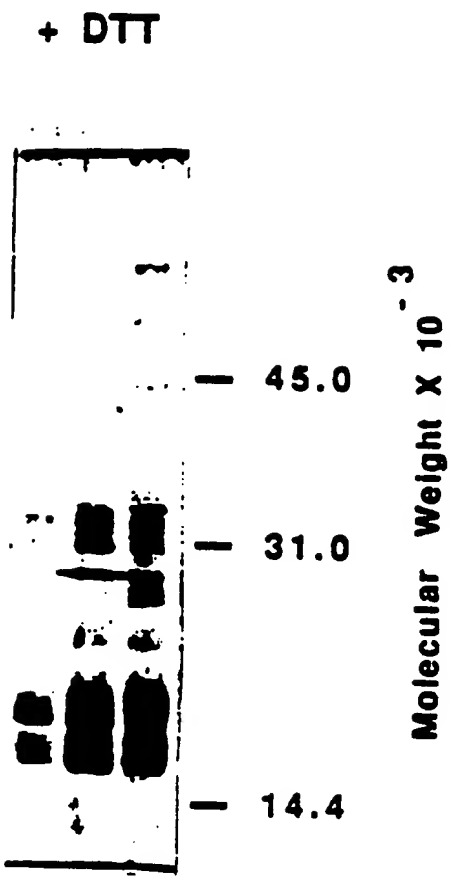


Fig. 11A

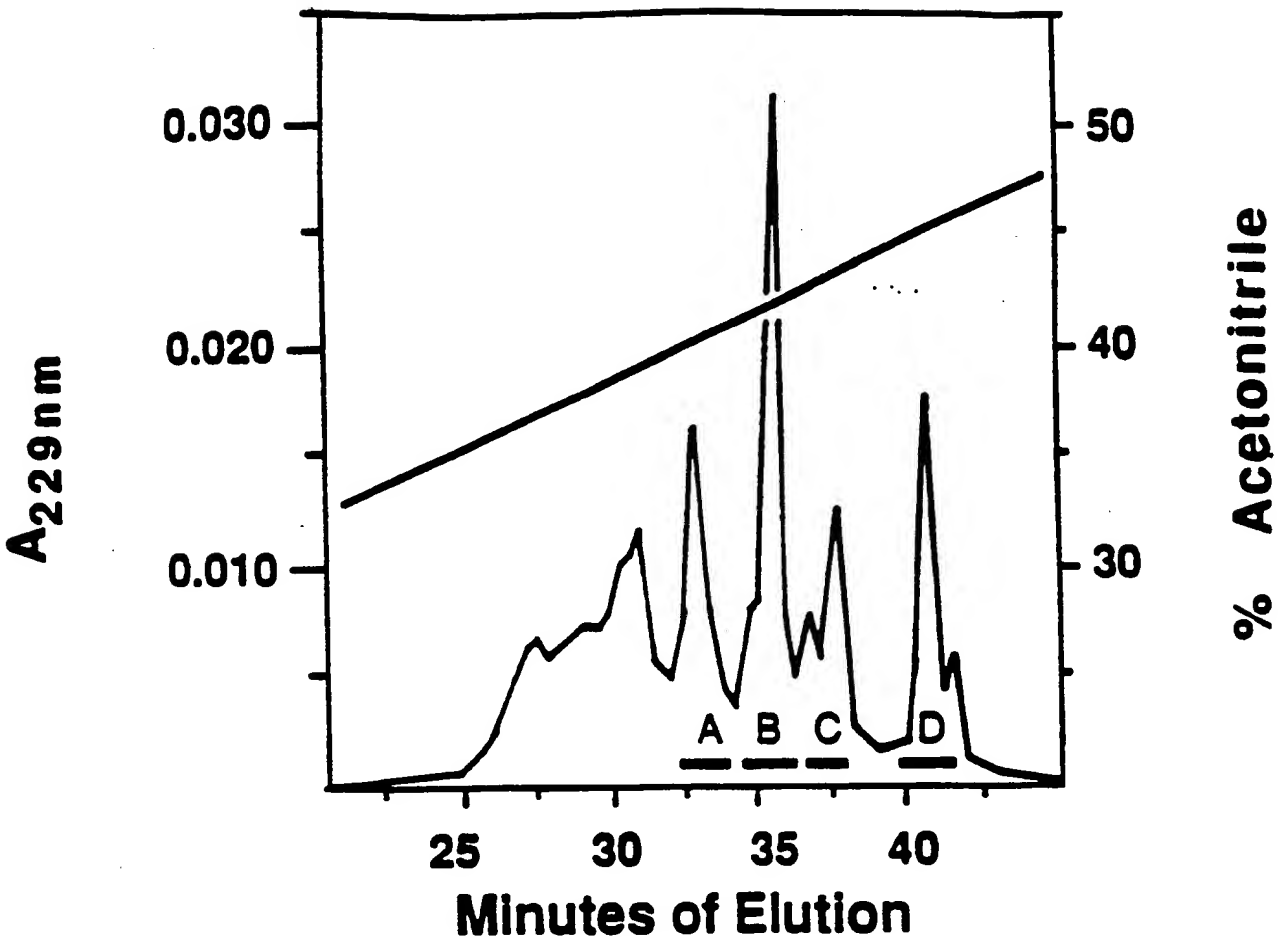


Fig. 11B

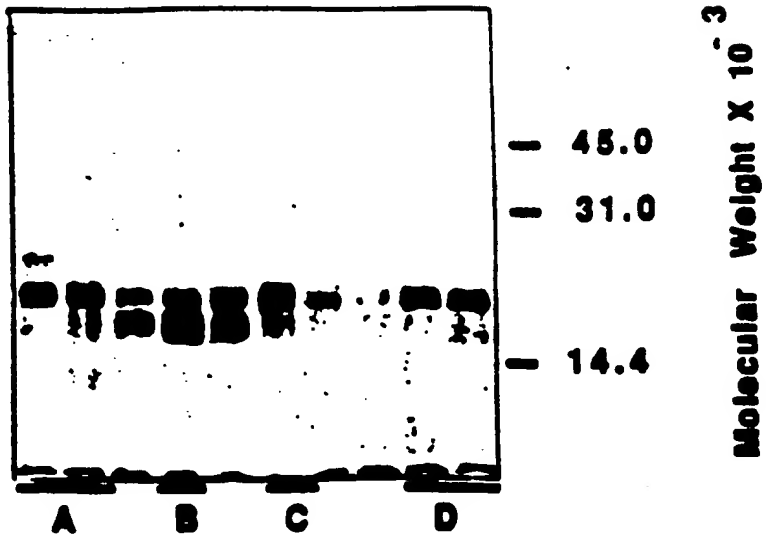


Fig. 12

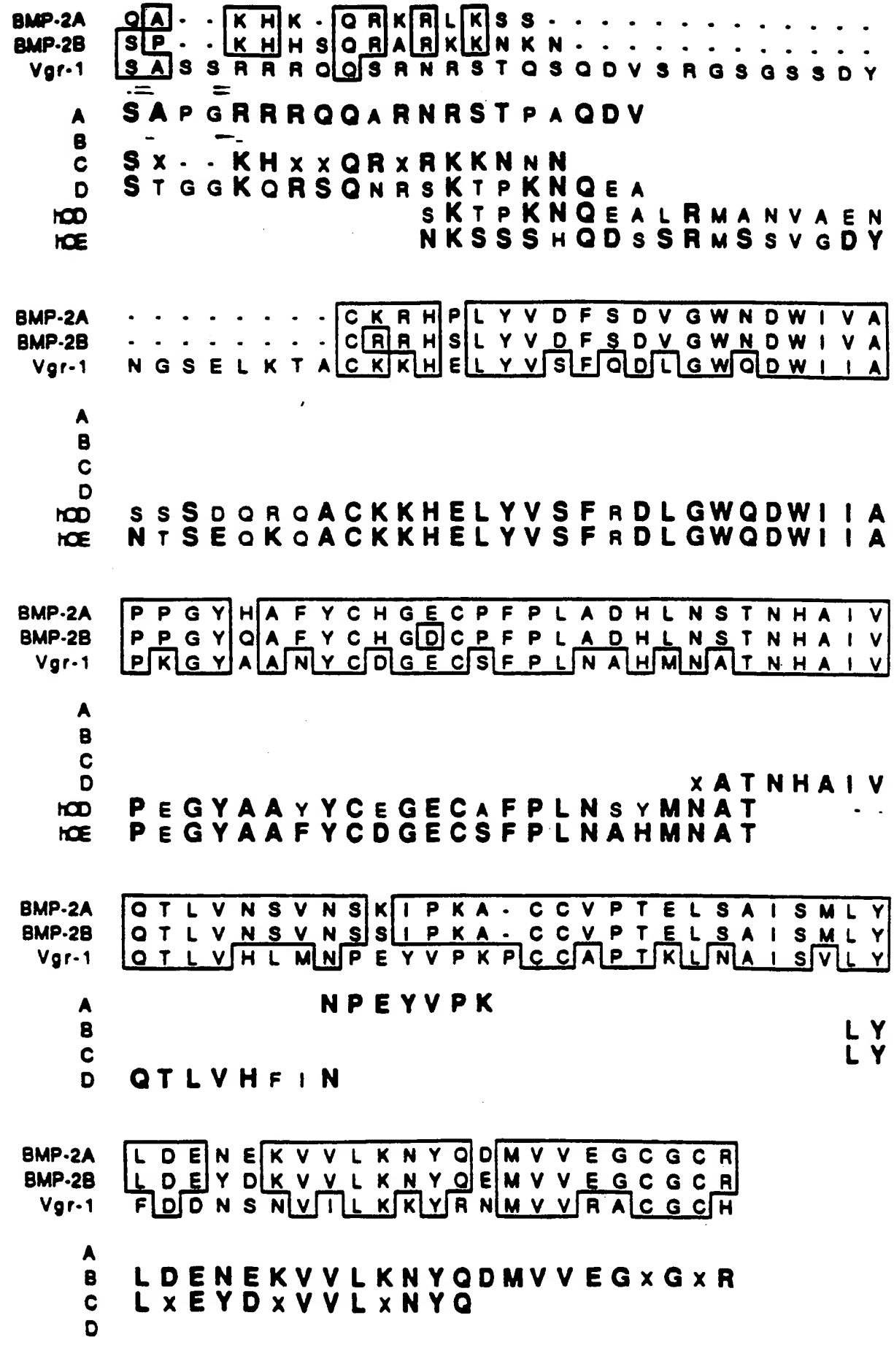


Fig. 13A

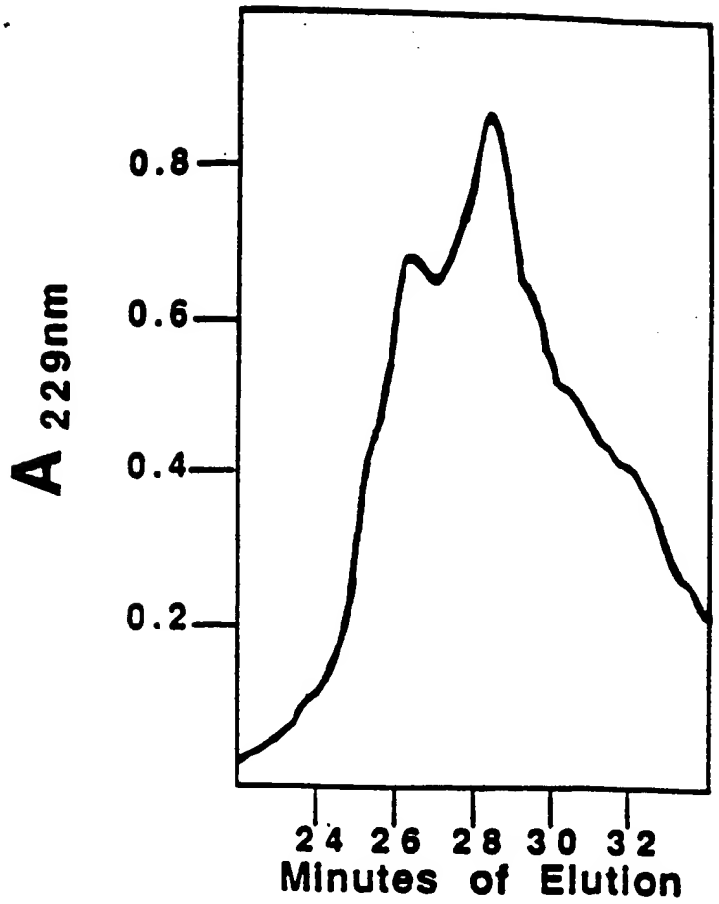


Fig. 13B

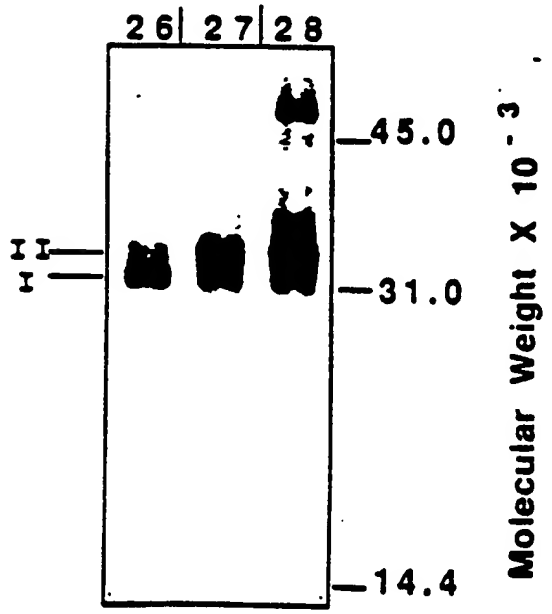


Fig. 14A

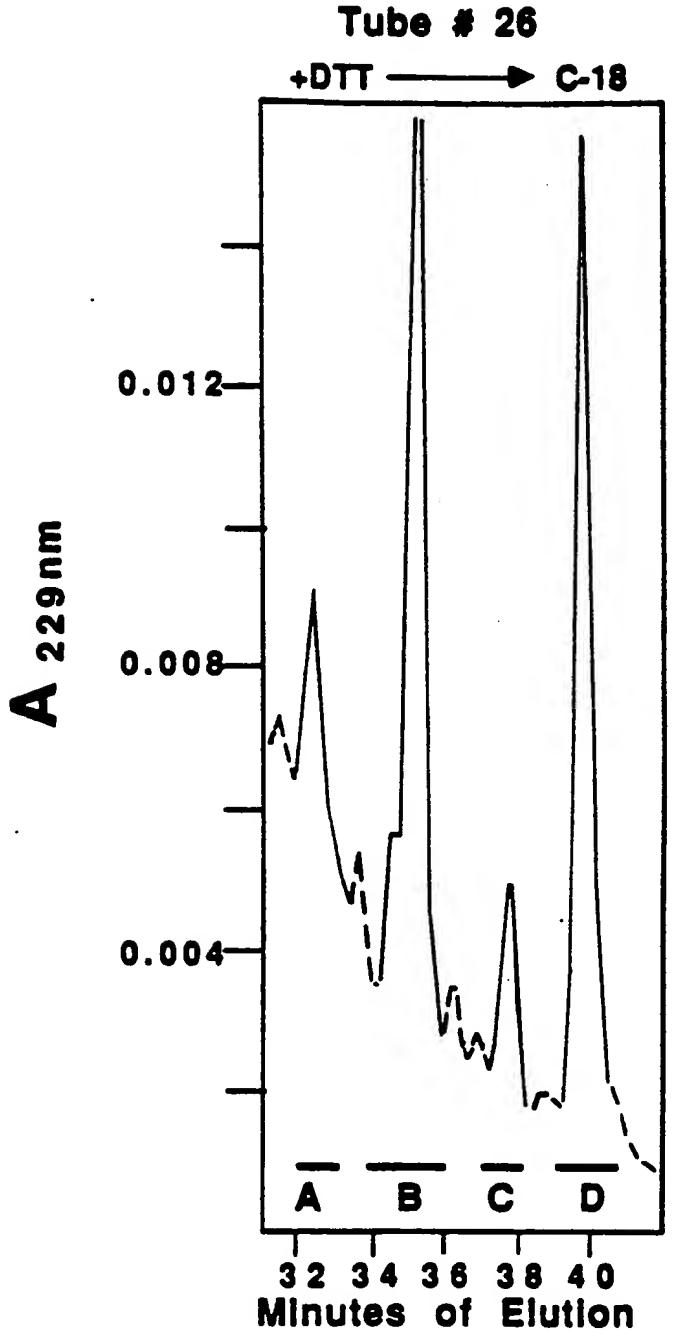


Fig. 14B

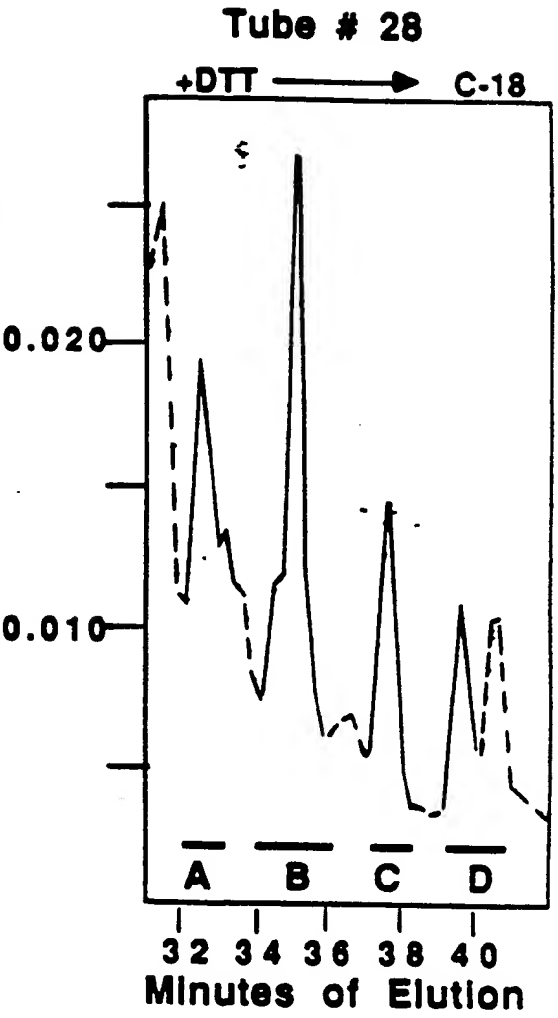


Fig. 15

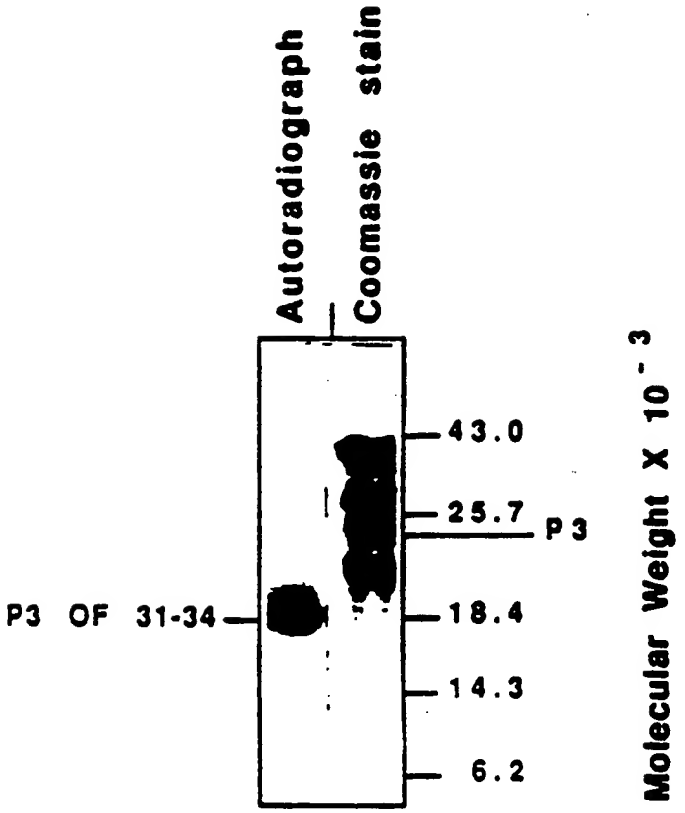

Autoradiograph
Coomassie stain
43.0
25.7
P3
18.4
14.3
6.2
P3 OF 31-34
Molecular Weight X 10 - 3

Fig. 16

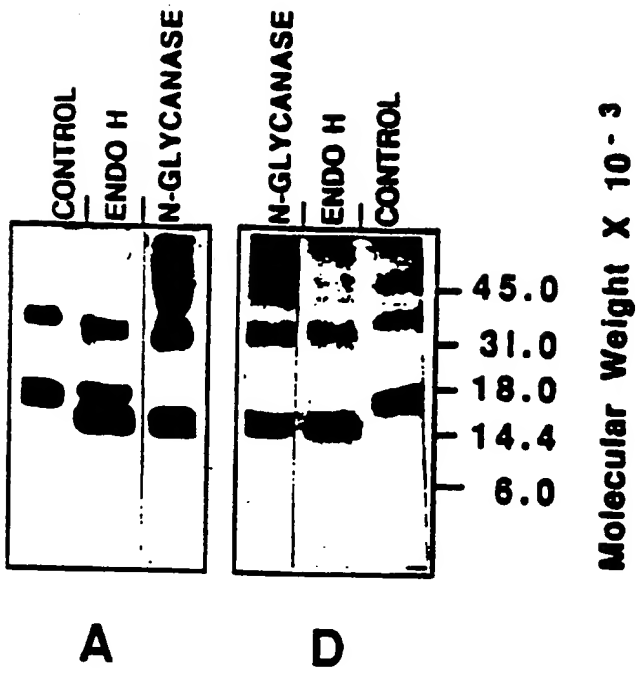

CONTROL
ENDO H
N-GLYCANASE
N-GLYCANASE
ENDO H
CONTROL
45.0
31.0
18.0
14.4
6.0
Molecular Weight X 10 - 3
A
D

Fig. 17

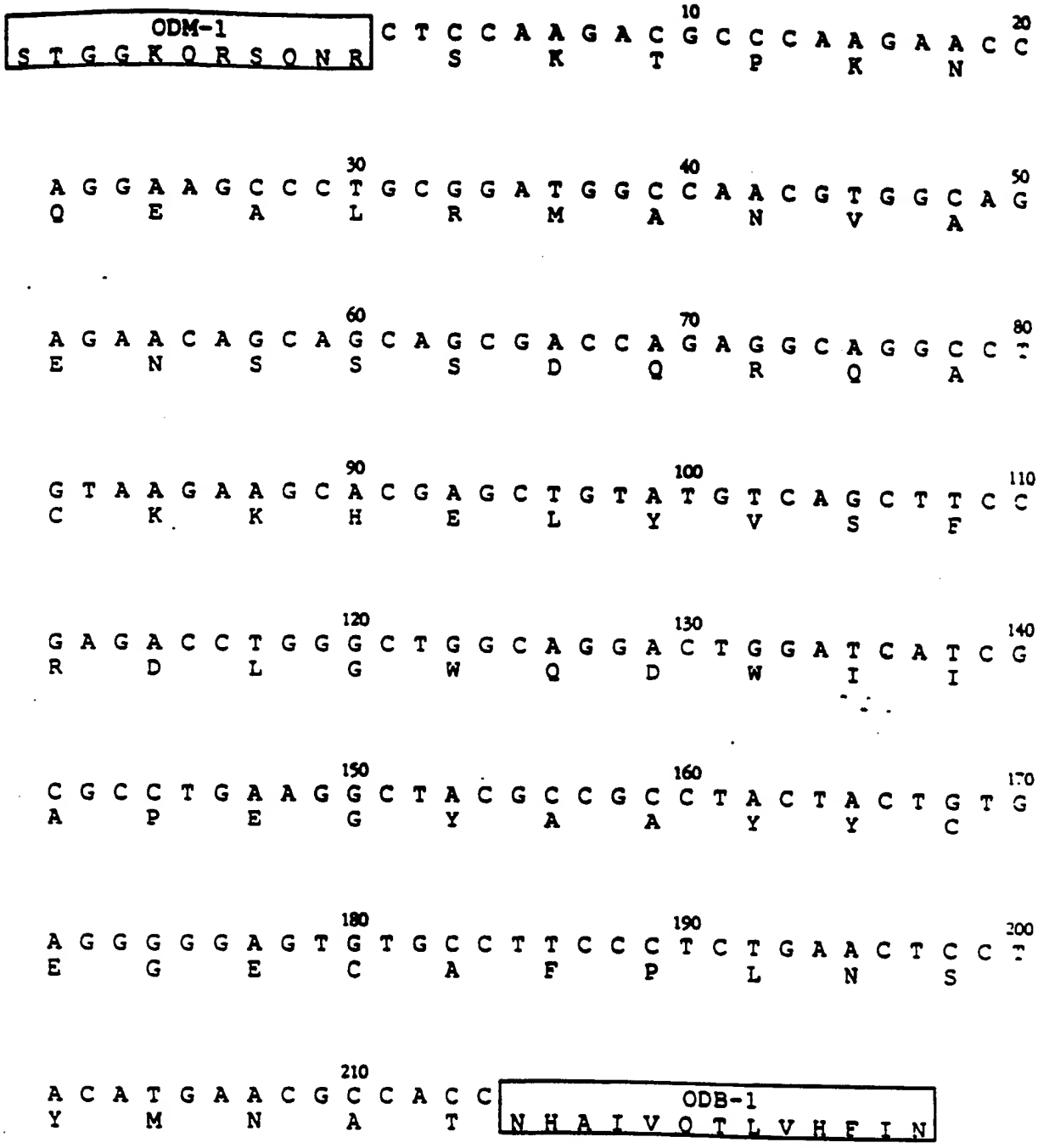
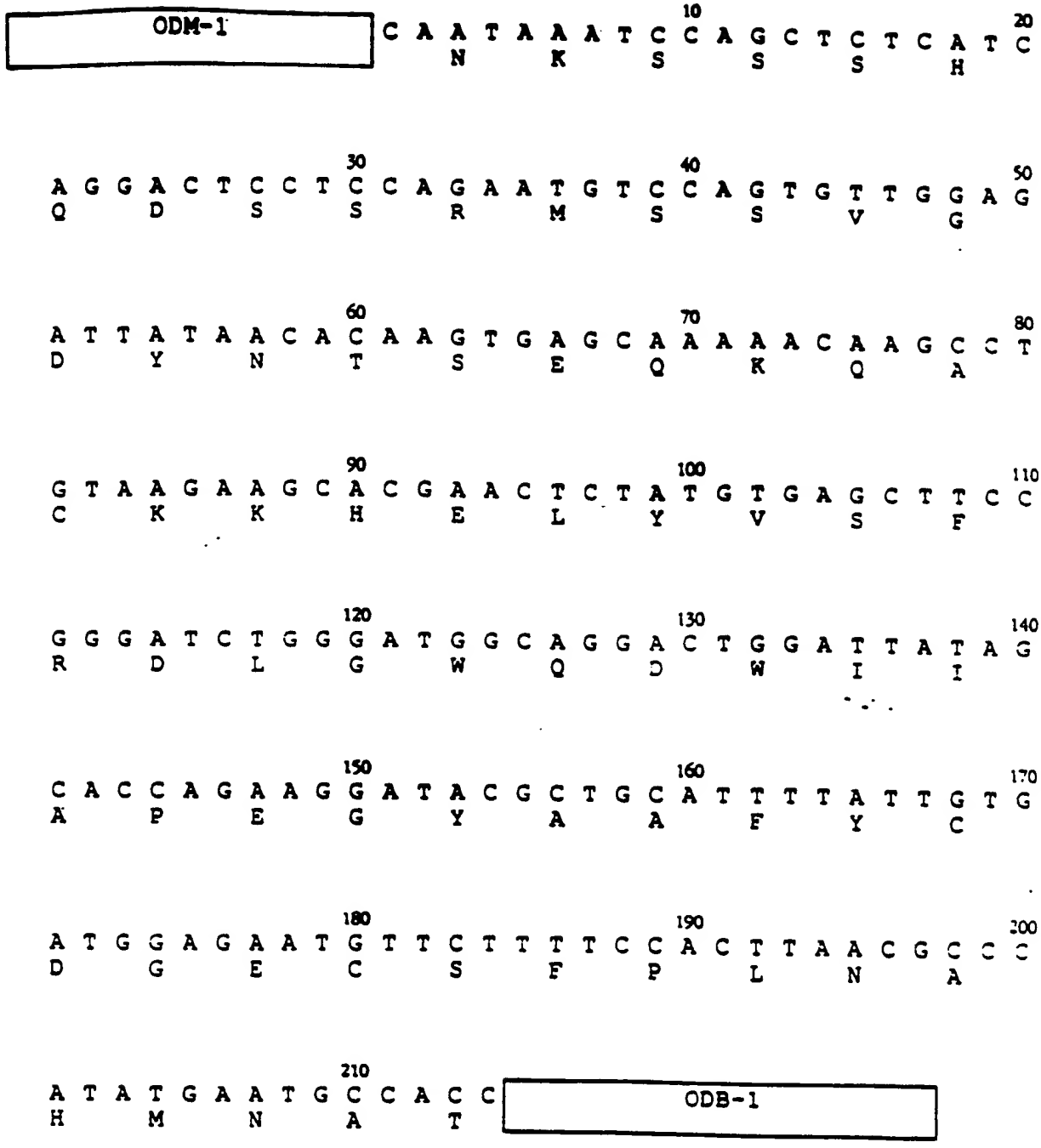


Fig. 18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N K S S S H
10 20
A G G A C T C C T C C A G A A T G T C C A G T G T T G G A G
Q D S S R M S S V G
30 40 50
A T T A T A A C A C A A G T G A G C A A A A A C A A G C C T
D Y N T S E Q K Q A
60 70 80
G T A A G A A G C A C G A A C T C T A T G T G A G C T T C C
C K K H E L Y V S F
90 100 110
G G G A T C T G G G A T G G C A G G A C T G G A T T A T A G
R D L G W Q D W I I
120 130 140
C A C C A G A A G G A T A C G C T G C A T T T T A T T G T G
A P E G Y A A F Y C
150 160 170
A T G G A G A A T G T T C T T T C C A C T T A A C G C C C
D G E C S F P L N A
180 190 200
A T A T G A A T G C C A C C
H M N A T
210
ODB-1

Fig. 19A

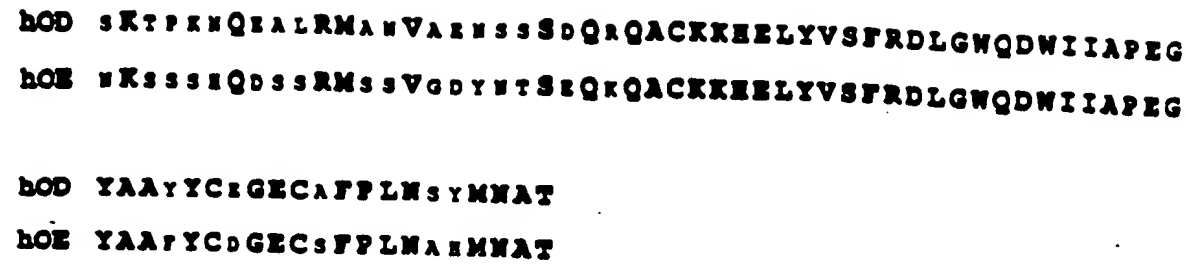
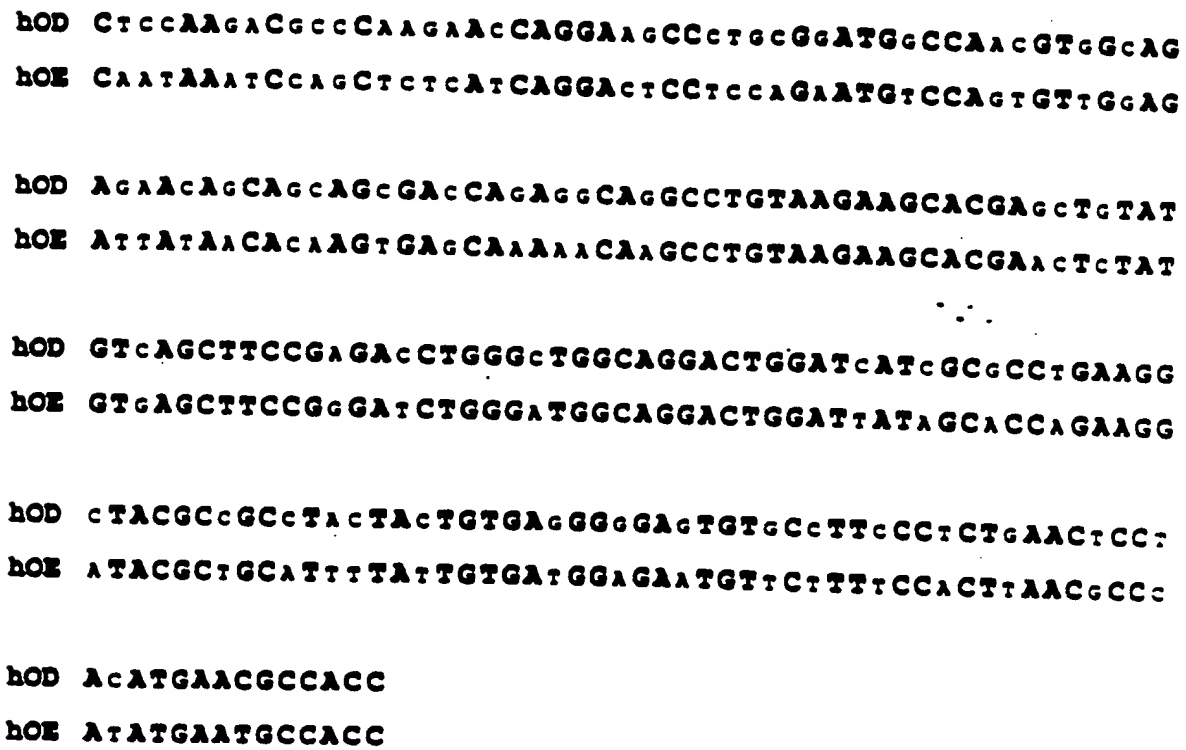


Fig. 19B



I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

INT Cl.4 A01N 63/02, A61K 35/32, A61K 37/12
U.S. Cl. 424/95, 530/350, 840

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
INT Cl.4	A01N 63/02, A61K 35/12, A61K, 35/32, A61K 37/12
U.S. Cl.	424/95, 530/350, 840

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
A	US, A, 4,627,982 (Seyedin et al.) 09 December 1986. See entire document.	1-16
A	US, A, 4,563,350 (Nathan et al.) 07 January 1986. See entire document.	1-16
A	US, A, 4,455,256 (Urist et al.) 19 June 1984. See entire document.	1-16
A	Clinical orthopaedics and related research, 171, 213-244, published December 1982, "The Nature ot Bone Morphogenetic Protein (BMP) Fractions Derived From Bovine Bone Matrix Gelatin," Mizutani et al. See entire document.	
A	Biomedical Research, 2(5), 466-471 published 1981, "Purification ot a Bone-Inducing Substance (osteogenic factor) from a Murine osteosarcoma", Takaoka et al., See entire document	1-16
A	EPA, 0169016, (Seyedin et al.), 16 July, 1984 See entire document	1-16

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
01 December 1989	22 JAN 1990
International Searching Authority	**Signature of Authorized Officer**
ISA/U.S.	Carlos Azpuru Carlos Azpuru

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers . because they relate to subject matter [2] not required to be searched by this Authority, namely:

2. ☐ Claim numbers , because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [3], specifically:

3. ☐ Claim numbers_____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

Telephone Practice: See attachment

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

1-16

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.